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(54) Title: M. TUBERCULOSIS RNA POLYMERASE ALPHA SUBUNIT

(57) Abstract

The present invention provides isolated nucleic acids encoding RNA polymerase alpha subunit from *M. tuberculosis*, vectors comprising the nucleic acids, cells comprising the vectors, and methods for producing *M. tuberculosis* alpha subunit. The invention also provides *in vitro* and *in vivo* methods for high–throughput screening to identify inhibitors of *M. tuberculosis* RNA polymerase.

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M. TUBERCULOSIS RNA POLYMERASE ALPHA SUBUNIT

Field of the Invention

The present invention relates to novel nucleic acids encoding RNA polymerase alpha subunit from *M. tuberculosis* and methods for use thereof.

Background of the Invention

The intracellular pathogen *Mycobacterium tuberculosis* is the causative agent of tuberculosis in humans and is responsible for millions of deaths worldwide each year (Bloom et al. *Science* 257:1055, 1992). The emergence of multidrug resistant forms of tuberculosis has mandated the development of new antibiotics effective against refractory *M. tuberculosis* strains. Novel drugs which, like rifampin (Vall-Spinosa et al., *N. Eng. J. Med.* 283: 616, 1970), may be capable of inhibiting the prokaryotic transciptional machinery, could contribute significantly to the development of therapies to combat *M. tuberculosis*.

The RNA polymerase of eubacteria consists of four subunits (α, β, β', and σ) and exists in two major forms: core enzyme (α_2, β, β') and holoenzyme (α_2, β, β' plus one of several σ subunits) (Chamberlin, in *RNA Polymerase*, Losick et al., eds., Cold Spring Harbor, 1976, pp. 17-67). The α subunit contains determinants for protein-protein

interactions with transcription activators and protein-DNA interaction with upstream elements (Ishihama, *Mol.Microbiol.* 6:3283, 1992; Russo et al., *J. Biol.Chem.* 267:14515, 1992; Ebright et al., *Curr.Opin.Genet.Dev.* 5:197, 1995). The amino terminal domain of α is also required for assembly of the multisubunit core RNA polymerase (Ishihama, *Adv. Biophys.* 14:1, 1981). The β subunit is essential for transcript initiation and elongation, which β' apparently functions in binding of the core enzyme to template DNA (Yura et al., *Ann.Rev.Genet.* 13:59, 1979). Core RNA polymerase is capable of RNA synthesis; however, σ is required for specific initiation of transcripts at promoter sequences (Gross et al., in *Transcriptional Regulation*, Cold Spring Harbor, 1992, pp. 129-176).

Discovery of inhibitors of *M. tuberculosis* RNA polymerase is hampered by a lack of information concerning components of the *M. tuberculosis* transcriptional apparatus, difficulties in obtaining sufficient yields of active enzymes for biochemical studies, and biosafety concerns. Establishment of an *in vitro* transcription system employing purified and reconstituted RNA polymerase would greatly advance efforts to identify new therapeutic agents active against tuberculosis.

Accordingly, there is a need in the art for compositions and methods utilizing cloned genes and purified proteins derived from *M. tuberculosis* RNA polymerase.

Summary of the Invention

The present invention is based on the isolation and characterization of DNA encoding the α subunit of RNA polymerase derived from *M. tuberculosis*. In one aspect, the invention provides a purified, isolated nucleic acid having the sequence shown in Figure 3. The invention also encompasses sequence-conservative and function-conservative variants of

this sequence. The invention also provides vectors comprising these sequences, and cells comprising the vectors.

In another aspect, the present invention provides a purified, isolated polypeptide encoded by the nucleic acid sequence shown in Figure 3, as well as function-conservative variants thereof. In one embodiment, the invention provides a purified α subunit further comprising a hexahistidine tag. The invention also provides purified, reconstituted core- and holoenzyme comprising the α subunit.

In yet another aspect, the invention provides *in vitro* methods for high-throughput screening to detect inhibitors of *M. tuberculosis* RNA polymerase. In one embodiment, the methods comprise:

a) providing a mixture comprising

(i) purified *M. tuberculosis* RNA polymerase and

(ii) a DNA template encoding a promoter sequence that is recognized by *M. tuberculosis* RNA polymerase;

b) incubating the mixture in the presence of test compounds to form test samples, and in the absence of test compounds to form control samples, under conditions that result in RNA synthesis in the control samples;...

c) measuring RNA synthesis directed by said *M. tuberculosis*-recognized promoter in the test and control samples; and

d) comparing the RNA synthesis detected in step (c) between the test and control samples. According to the invention, an RNA polymerase inhibitor is a test compound that causes a reduction in RNA synthesis directed by the *M. tuberculosis*-recognized promoter measured in the test sample relative to RNA synthesis measured in the

control sample. In a preferred embodiment, the purified RNA polymerase used in practicing this method comprises recombinant subunits which are reconstituted to form an enzymatically active holoenzyme. In another embodiment, the ability of test compounds to bind to purified α subunit is monitored.

In yet another aspect, the invention provides *in vivo* methods for high-throughput screening to detect inhibitors of *M. tuberculosis* RNA polymerase. The methods are carried out by the steps of:

a) providing a non-mycobacterial bacterial strain, preferably *E. coli*, that

(i) has been transformed with a DNA template encoding a promoter sequence that is recognized by *M. tuberculosis* RNA polymerase, and

(ii) expresses enzymatically active *M. tuberculosis* RNA polymerase (e.g., α_2, β, β' plus one of several σ subunits);

b) incubating the bacterial strain of (a) in the presence of test compounds to form test samples, and in the absence of test compounds to form control samples;

c) measuring RNA synthesis directed by said *M. tuberculosis*-recognized promoter in the test and control samples; and

d) comparing the RNA synthesis directed by said *M. tuberculosis*-recognized promoter detected in step (c) between the test and control samples. According to the invention, an RNA polymerase inhibitor is a test compound that causes a reduction in RNA synthesis measured in the test sample relative to RNA synthesis measured in the control sample..

These and other aspects of the present invention will be apparent to those of ordinary skill in the art in light of the present specification and appended claims.

Brief Description of the Drawings

Figure 1 is a graphic illustration comparing the deduced amino acid sequence of the *M. tuberculosis rpoA* gene fragment and corresponding regions of α subunits of *B. subtilis* and *E. coli*. Mt, *M. tuberculosis*; Bs, *B. subtilis*; Ec, *E. coli*. Dashed lines denote conserved segments of *B. subtilis* and *E. coli* proteins for which oligonucleotides were designed.

Figure 2A is a photographic illustration of a Southern blot analysis of *rpoA* hybridizing cosmid clones. Four different positive clones (designated IIA5, IIA3, ID9, and IC5) isolated from an *M. tuberculosis* cosmid library were digested with *Not*I and hybridized with an *rpoA* gene probe. Sizes of DNA markers (λ EcoT141, Amersham) are indicated in kb.

Figure 2B is a photographic illustration of a Southern blot analysis of *rpoA* hybridizing cosmid clones, showing structural conservation of genomic and cosmid-borne *rpoA* sequences. Purified cosmid IC5 DNA (lanes 1-5) or *M. tuberculosis* H37Rv genomic DNA (lanes 7-11) were digested with the indicated restriction enzymes and hybridized with an *rpoA* probe. Sizes of DNA markers (λ EcoT141, Amersham) are indicated in kb.

Figure 3 shows the nucleotide and deduced amino acid sequences of the *M. tuberculosis* H37Rv *rpoA* gene. RBS denotes a potential ribosome binding site. The putative start and stop codons of the *rpoA* gene product are at 85-87 and 1126-1128, respectively.

Figure 4 is a graphic illustration comparing the inferred amino acid sequence of the *M. tuberculosis* H37Rv α subunit with sequences of α subunits from other bacteria. *B. subtilis* (Bs), *M. genitalium* (Mg), *E. coli* (Ec), *H. influenza* (Hi), *B. pertusis* (Bp), *S.*

typhimurium (St), and *C. trachomitis* (Ct). Shading indicates identical amino acid residues. Amino acid sequence alignments were performed using MegAlign (DNAStar).

Figure 5 is a photographic illustration of an SDS-polyacrylamide gel electropherogram depicting the expression and purification of *M. tuberculosis* α subunit in *E. coli*. Lane 1 shows molecular mass markers (kDa); lanes 2 and 3 show total proteins from uninduced and IPTG-induced cultures, respectively, of *E. coli* harboring pJH37; and lane 4 shows purified *M. tuberculosis* α subunit (approximately 38 kDa), which is indicated by an arrow.

Figure 6 is a photographic illustration of an SDS-polyacrylamide gel electropherogram showing the reconstitution and purification of core- and holo- RNA polymerase from *M. tuberculosis*. Lanes 1 and 6 show molecular mass markers (kDa); lane 2 shows α-histidine-containing core enzyme; lane 3 shows a histidine-containing holoenzyme; lane 4 shows N-terminal histidine-tagged primary sigma; and lane 5 shows purified *E. coli* holoenzyme.

Figure 7 is a graphic illustration of *in vitro* transcription reactions employing reconstituted purified *M. tuberculosis* RNA polymerase holoenzyme and T101 and T125 promoter-containing DNA templates.

Detailed Description of the Invention

All patent applications, patents and literature references cited herein are hereby incorporated by reference in their entirety. In the case of inconsistencies the present disclosure will prevail.

The present invention is based on the isolation of a fragment of the *M. tuberculosis rpoA* gene, encoding the α subunit of RNA polymerase. As described in Example 1 below, the fragment was isolated by the polymerase chain reaction (PCR) using primers directed against regions of α that are highly conserved among Gram-positive and Gram-negative bacteria. The amplified DNA was utilized as a hybridization probe to recover the entire *rpoA* gene from a cosmid library of genomic DNA from virulent *M. tuberculosis* strain H37RV. Nucleotide sequencing indicated that the 1044 bp *M. tuberculosis rpoA* open reading frame (ORF) encodes a protein of 347 amino acids which shows significant structural similarity to the α subunits of diverse bacterial species with greatest identity to the *B. subtilis* α protein (Figure 1).

Though structurally conserved throughout most of its length, the inferred amino acid sequence of the *M. tuberculosis* α subunit diverged from that of *B. subtilis* and other bacteria at its extreme C-terminus. Interestingly, studies on *E. coli* RNA polymerase subunits have indicated that the C-terminal 94 residues of α function in interactions with certain transcriptional regulatory proteins, while the N-terminal domain may play a more highly conserved role in facilitating the core assembly (Ishihama, *Mol.Microbiol.* **6**:3283, 1992; Russo et al., *J.Biol.Chem.* **267**:14515, 1992).

The *B. subtilis rpoA* gene is part of a large operon that is cotranscribed with genes for initiation factor 1 and ribosomal proteins B, S13, S11, and L17; and, unlike the pattern of gene organization seen in *E. coli*, the *B. subtilis rpoA* gene cluster is genetically linked to the *rpoB-C* operon encoding RNA polymerase components β and β' (Boylan et al., *J. Bacteriol.* **171**:2553, 1992).

In practicing the present invention, many techniques in molecular biology, microbiology, recombinant DNA, and protein biochemistry such as these explained fully in, for example, Sambrook *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; *DNA Cloning: A Practical Approach*, Volumes I and II, 1985 (D.N. Glover ed.); *Oligonucleotide Synthesis*, 1984, (M.L. Gait ed.); *Transcription and Translation*, 1984 (Hames and Higgins eds.); *A Practical Guide to Molecular Cloning*; the series, *Methods in Enzymology* (Academic Press, Inc.); and *Protein Purification: Principles and Practice*, Second Edition (Springer-Verlag, N.Y.), may be used.

The present invention encompasses nucleic acid sequences encoding the α subunit of *M. tuberculosis* RNA polymerase, enzymatically active fragments derived therefrom, and related sequences. As used herein, a nucleic acid that is "derived from" a sequence refers to a nucleic acid sequence that corresponds to a region of the sequence, sequences that are homologous or complementary to the sequence, and "sequence-conservative variants" and "function-conservative variants". Sequence-conservative variants are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position. Function-conservative variants are those in which a given amino acid residue in the α subunit has been changed without altering the overall conformation and function of the polypeptide, including, but not limited to, replacement of an amino acid with one having similar physico-chemical properties (such as, for example, acidic, basic, hydrophobic, and the like). Fragments of the α subunit that retain enzymatic activity can be identified according to the methods described herein, such as e.g., expression in *E. coli*

followed by enzymatic assay of the cell extract or by purification and reconstitution of the purified polypeptide into an enzymatically active holoenzyme.

The nucleic acids of the present invention include purine- and pyrimidine-containing polymers of any length, either polyribonucleotides or polydeoxyribonucleotides or mixed polyribo-polydeoxyribo nucleotides. This includes single- and double-stranded molecules, i.e., DNA-DNA, DNA-RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone. This also includes nucleic acids containing modified bases. The nucleic acids may be isolated directly from cells. Alternatively, PCR can be used to produce the nucleic acids of the invention, using either chemically synthesized strands or genomic material as templates. Primers used for PCR can be synthesized using the sequence information provided herein and can further be designed to introduce appropriate new restriction sites, if desirable, to facilitate incorporation into a given vector for recombinant expression.

The nucleic acids of the present invention may be flanked by natural *M. tuberculosis* regulatory sequences, or may be associated with heterologous sequences, including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5`- and 3`- noncoding regions, and the like. The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, and internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.). Nucleic acids may contain one or more additional covalently linked moieties, such as, for example, proteins (e.g., nucleases,

toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (e.g., acridine, psoralen, etc.), chelators (e.g., metals, radioactive metals, iron, oxidative metals, etc.), and alkylators. The nucleic acid may be derivatized by formation of a methyl or ethyl phosphotriester or an alkyl phosphoramidate linkage. Furthermore, the nucleic acid sequences of the present invention may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

The invention also provides nucleic acid vectors comprising the disclosed α subunit sequences or derivatives or fragments thereof. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. Non-limiting examples include pKK plasmids (Clontech), pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), or pRSET or pREP (Invitrogen, San Diego, CA), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, e.g. antibiotic resistance, and one or more expression cassettes. Suitable host cells may be transformed/transfected/infected as appropriate by any suitable method including electroporation, $CaCl_2$ mediated DNA uptake, fungal infection, microinjection, microprojectile, or other established methods.

Appropriate host cells include bacteria, archebacteria, fungi, especially yeast, and plant and animal cells, especially mammalian cells. Of particular interest are *E. coli, B. subtilis, Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Schizosaccharomyces pombi, SF9* cells, C129 cells, 293 cells, *Neurospora*, and CHO cells, COS cells, HeLa cells, and

immortalized mammalian myeloid and lymphoid cell lines. Preferred replication systems include M13, ColE1, SV40, baculovirus, lambda, adenovirus, and the like. A large number of transcription initiation and termination regulatory regions have been isolated and shown to be effective in the transcription and translation of heterologous proteins in the various hosts. Examples of these regions, methods of isolation, manner of manipulation, etc. are known in the art. Under appropriate expression conditions, host cells can be used as a source of recombinantly produced Mycobacterial-derived peptides and polypeptides.

Advantageously, vectors may also include a transcription regulatory element (i.e., a promoter) operably linked to the α subunit portion. The promoter may optionally contain operator portions and/or ribosome binding sites. Non-limiting examples of bacterial promoters compatible with *E. coli* include: *trc* promoter, β-lactamase (penicillinase) promoter; lactose promoter; tryptophan (trp) promoter; arabinose BAD operon promoter; lambda-derived Pl promoter and N gene ribosome binding site; and the hybrid tac promoter derived from sequences of the trp and lac UV5 promoters. Non-limiting examples of yeast promoters include 3-phosphoglycerate kinase promoter, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, galactokinase (GALI) promoter, galactoepimerase promoter, and alcohol dehydrogenase (ADH) promoter. Suitable promoters for mammalian cells include without limitation viral promoters such as that from Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus (ADV), and bovine papilloma virus (BPV). Mammalian cells may also require terminator sequences and poly A addition sequences, and enhancer sequences which increase expression may also be included. Sequences which cause amplification of the gene may also be desirable. Furthermore, sequences that facilitate secretion of the recombinant

product from cells, including, but not limited to, bacteria, yeast, and animal cells, such as secretory signal sequences and/or prohormone pro region sequences, may also be included.

Nucleic acids encoding wild-type or variant α subunit polypeptides may also be introduced into cells by recombination events. For example, such a sequence can be introduced into a cell, and thereby effect homologous recombination at the site of an endogenous gene or a sequence with substantial identity to the gene. Other recombination-based methods, such as non-homologous recombinations or deletion of endogenous genes by homologous recombination, may also be used.

α subunit-derived polypeptides according to the present invention, including function-conservative variants, may be isolated from wild-type or mutant *M. tuberculosis* cells, or from heterologous organisms or cells (including, but not limited to, bacteria, fungi, insect, plant, and mammalian cells) into which an α subunit-derived protein-coding sequence has been introduced and expressed. Furthermore, the polypeptides may be part of recombinant fusion proteins (see, e.g., Example 2 below). Alternatively, polypeptides may be chemically synthesized by commercially available automated procedures, including, without limitation, exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis.

"Purification" of an α subunit polypeptide refers to the isolation of the polypeptide in a form that allows its enzymatic activity to be measured without interference by other components of the cell in which the polypeptide is expressed. Methods for polypeptide purification are well-known in the art, including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, and countercurrent distribution. For some purposes,

it is preferable to produce the polypeptide in a recombinant system in which the protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine sequence. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against the α subunit or against peptides derived therefrom can be used as purification reagents. Other purification methods are possible.

The isolated polypeptides may be modified by, for example, phosphorylation, sulfation, acylation, or other protein modifications. They may also be modified with a label capable of providing a detectable signal, either directly or indirectly, including, but not limited to, radioisotopes and fluorescent compounds.

Screening Methods to Identify Anti-tuberculosis Agents

The methods and compositions of the present invention can be used to identify compounds that inhibit the function of *M. tuberculosis* RNA polymerase and thus are useful as anti-tuberculosis agents. This is achieved by providing enzymatically active recombinant α subunit according to the present invention, in combination with other components of RNA polymerase, in a context in which the inhibitory effects of test compounds can be measured. Alternatively, the ability of test compounds to bind to purified α subunit is monitored, using methods well-known in the art. Test compounds identified using the methods of the invention encompass those that interfere with the enzymatic activity of RNA polymerase and/or disrupt the interaction of the α subunit with transcriptional activators. Thus, the compounds may function as general or gene-specific transcriptional inhibitors.

In a preferred embodiment, recombinant *M. tuberculosis* RNA polymerase subunits (α, β, β' plus one of several σ subunits) are purified in milligram quantities from *E. coli* cultures by affinity methods utilizing a hexahistidine tagged α subunit. Enzymatically active holoenzyme is reconstituted using these components (see, e.g., Example 2 below). The active polymerase is then incubated in the presence of test compounds to form test mixtures, and in the absence of test compounds to form control mixtures. *In vitro* transcription is then carried out using a DNA template containing appropriate promoter sequences (see, e.g., Example 2 below).

In another embodiment, *M. tuberculosis* RNA polymerase subunits (α_2, β, β' plus one of several σ subunits) are co-expressed in *E. coli* or another surrogate bacterial cell, in conjunction with an appropriate promoter functionally linked to a reporter gene. The ability of test compounds to differentially inhibit *M. tuberculosis* RNA polymerase is then assessed by measuring the amount and/or activity of the reporter gene product.

M. tuberculosis promoters useful in practicing the invention include without limitation: P1 or P2 hsp 60 promoters (Stover et al., *Nature* **351**:456, 1991); cpn-60 promoter (Kong et al., *Proc.Natl.Acad.Sci.USA* **90**:2608, 1993); 85A antigen promoter (Kremer, *J. Bacteriol.* **177**:642, 1995); PAN promoter (Murray et al., *Mol. Microbiol.* **6**:3331, 1992); 16S RNA promoter (Ji et al., *Microbiol.* **140**: 2829, 1994); and askβ promoter (Cirillo et al., *Mol. Microbiol.* **11**:629, 1994). Useful reporter genes include without limitation xylE (Curcic et al., *Mol. Microbiol* **13**:1057, 1994); CAT (Das Gupta et al., *J. Bacteriol.* **175**:5186, 1993); luciferase (Cooksey et al., *Antimicrob.Agts.Chemother.* **37**:1348, 1993); green fluorescent protein (Dhadayuthap et al., *Mol. Microbiol.* **17**:901, 1995); and lacZ (Silhavy et al., *Microbiol.Rev.* **49**:398, 1985).

It will be understood that the present invention encompasses *M. tuberculosis* RNA polymerases containing any appropriate σ factor, which is used in conjunction with a particular promoter that is recognized by RNA polymerase containing that σ factor. Non-limiting examples of useful σ factors include σ^A, σ^B, and σ^F (Doushkan et al., 1995; DeMaio et al., *Gene* **165**:67, 1995). The invention also encompasses the identification of additional promoters that are recognized by a particular σ subunit. This is achieved by providing a library of random *M. tuberculosis* gene fragments cloned upstream of an appropriate reporter gene (see above). The library is transformed into *M. tuberculosis* or *M. smegmatis* and reporter gene expression is measured. Alternatively, the library is transformed into another bacterial cell, such as, e.g., *E. coli*, which expresses *M. tuberculosis* RNA polymerase core subunits as well as a σ subunit of interest and cognate promoters that drive reporter gene expression. In yet another embodiment, expression of an *M. tuberculosis* σ factor confers new recognition properties on *E. coli* RNA polymerase and permits isolation of promoters utilized specifically by a particular *M. tuberculosis* σ subunit.

Preferably, both *in vitro* and *in vivo* screening methods to detect *M. tuberculosis*-specific RNA polymerase inhibitors are adapted to a high-throughput format, allowing a multiplicity of compounds to be tested in a single assay. Such inhibitory compounds may be found in, for example, natural product libraries, fermentation libraries (encompassing plants and microorganisms), combinatorial libraries, compound files, and synthetic compound libraries. For example, synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich Chemical Company, Inc. (Milwaukee, WI). Alternatively,

libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from, for example, Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle et al., *TibTech* 14:60, 1996), preferably using automated equipment, to allow for the simultaneous screening of a multiplicity of test compounds.

Useful anti-tuberculosis compounds are identified as those test compounds that decrease tuberculosis-specific transcription. Once a compound has been identified by the methods of the present invention as an RNA polymerase inhibitor, *in vivo* and *in vitro* tests may be performed to further characterize the nature and mechanism of the inhibitory activity. For example, classical enzyme kinetic plots can be used to distinguish, e.g., competitive and non-competitive inhibitors.

Compounds identified as RNA polymerase inhibitors using the methods of the present invention may be modified to enhance potency, efficacy, uptake, stability, and suitability for use in pharmaceutical formulations, etc. These modifications are achieved and tested using methods well-known in the art.

The present invention is further described in the following examples which are intended to further describe the invention without limiting the scope thereof.

Example 1: Isolation and Analysis of the *M. tuberculosis rpoA* Gene

The following experiments were performed in order to clone the gene encoding *M. tuberculosis* RNA polymerase α subunit. In these experiments, the following materials and methods were used.

A. Materials and Methods

PCR amplification: *M. tuberculosis* H37Rv genomic DNA (1 ng) was amplified in a 100 µl reaction containing: 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 500 mM KCl, 2.5 U Taq polymerase (Beohringer-Mannheim), 0.2 mM dNTPs and oligonucleotide primers (1 µM each): 5'-CTACGCAAGCAGGGTCCGGGTGAG-3' and 5'-CTCACCCGGACCCTGCTTGCGTAG-3'. PCR was performed in an Omnigene thermocycler with 35 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 1 min. The amplification product was gel-purified and ligated into vector pCR2.1 (Invitrogen).

Cosmid hybridizations: A transducing lysate of a cosmid library of *M. tuberculosis* H37Rv genomic DNA in vector pYA3060 was used. Cosmid-bearing *E. coli* Φ2819T (Jacobs et al., *Infec.Immun.* **52**:101, 1986) colonies representing roughly five genomic equivalents were individually picked to wells of sterile 96-well microliter dishes and propagated at 30°C in Luria broth containing ampicillin at 30 µg/ml and thymidine at 50 µg/ml. Colonies were grown overnight at room temperature on the above media as nylon filter replicas of the library. Filters were processed for colony hybridization by standard methods and probe hybridizations performed as described below. Cosmid DNAs were purified using maxiprep columns (Qiagen).

Southern blot analysis: Restriction enzyme digests of *M. tuberculosis* H37Rv chromosomal or cosmid DNAs were resolved by electrophoresis on 1% TAE-agarose gels and blotted to nylon filters using standard methods. Probe labeling and hybridizations were performed using the Gene Images hybridization system (Amersham) essentially as described by the supplier except that hybridizations and washes were carried out at 70°C.

DNA sequencing and analysis: Plasmid templates for nucleotide sequencing were purified using RPM miniprep kits (Bio101). PCR cycle sequencing (ABI Prizm) was carried out with an Applied Biosystems automated sequencer.

B. Strategy for *M. tuberculosis rpoA* gene isolation

A fragment of the *M. tuberculosis rpoA* gene was isolated by PCR, using oligonucleotide primers homologous to defined regions of α that are conserved among Gram-positive and Gram-negative bacterial species. Amino acid sequence alignments of the α subunits of *B. subtilis* and *E. coli* revealed several short regions of identity clustered primarily within the N-terminal half of α implicated in association with RNA polymerase core subunits. Primers corresponding to conserved α segments LRRILLSS and VTAADITHD (amino acids 39-46 and 106-114, respectively of the *B. subtilis* subunit) specifically amplified a DNA fragment of anticipated size (228 bp) using *M. tuberculosis* H37Rv genomic DNA as a template. To confirm that the amplified DNA contained *rpoA* sequences, the 228 bp fragment was subcloned and a partial nucleotide sequence determined. The deduced amino acid sequence of the PCR product showed significant homology to the α subunits of other bacteria (Figure 1), confirming its identity as an *rpoA* gene fragment. One ORF of the 228 bp fragment displayed high degree of identity to corresponding intervals of the *B. subtilis* (61%)

and *E. coli* (55%) α subunits. The *M. tuberculosis rpoA*-derived sequence had an overall G+C content of 60% while the G+C content for bases occupying the third position of each codon increased to 76%, values in good agreement with compositions of other *M. tuberculosis* genes (Weiden et al., in *Tuberculosis*, Little, Brown, 1996, pp. 211-222).

C. Southern blot analysis and isolation of the entire *M. tuberculosis rpoA* gene

To establish that the cloned 228 bp fragment was mycobacterial in origin, Southern blot analysis of restriction enzyme digests of *M. tuberculosis* H37Rv genomic DNA was performed. The PCR probe detected unique restriction fragments in chromosomal digests (Figure 2B). These data are consistent with a single copy of the *rpoA* gene in the *M. tuberculosis* chromosome (two bands of hybridization observed in the *Eco*RI digest result from the presence of an *Eco*RI site within the 228 bp probe sequence). The full-length *rpoA* gene was obtained from a cosmid library of *M. tuberculosis* H37Rv genomic fragments using the 228 bp PCR probe. Screening of approximately 600 cosmid-bearing *E. coli* colonies (representing roughly 5 genome equivalents) with the *rpoA* gene fragment yielded 4 positive clones. Restriction analysis indicated that the 4 isolates differed from one another with respect to total insert size, though all shared a roughly 10 kb *Not*I fragment which hybridized to the *rpoA* gene probe (Figure 2A). One *rpoA*-hybridizing cosmid clone, designated IC5, was chosen for further analysis. Southern blotting of IC5 DNA digested with a panel of restriction enzymes confirmed that the no gross structural rearrangements of the *rpoA* gene had occurred during cloning (Figure 2B). The 1.4 kb SacII and 1.3 kb SalI *rpoA*-hybridizing fragments of cosmid IC5 were subcloned into vector pSKII+ prior to nucleotide sequencing.

D. Sequence analysis of the *M. tuberculosis rpoA* gene

Nucleotide sequencing was performed on plasmid subclones and on cosmid IC5 DNA. Nucleotide and deduced amino acid sequences of the *rpoA* gene of *M. tuberculosis* are shown in Figure 3. The sequence encodes a 1044 bp ORF which has an overall G+C composition of 63% (85% for bases occupying the codon third position). Assuming that the ATG at position 85-87 serves as the initiator codon, the ORF is expected to encode a protein of 347 amino acids. A strong match with the consensus sequence for an *M. tuberculosis* ribosome binding site (GAAAGGA), (Novick, in *Tuberculosis*, Little, Brown, 1996, pp. 187-198) is positioned just upstream of the putative ATG codon. Examination of more than 80 bp of nucleotide sequence upstream of the translation start site did not reveal regions of exact identity with prokaryotic promoter sites. Among α subunits studied in other bacterial species, the deduced amino acid sequence of the 347 residue *M. tuberculosis* protein displayed greatest similarity (48% identity) to the α subunit of the Gram-positive sporulating bacterium *B. subtilis* (Figure 4).

Example 2: Expression of *M. tuberculosis* RNA Polymerase α Subunit and Reconstitution of Active *M. tuberculosis* RNA Polymerase

The following experiments were performed to purify *M. tuberculosis* RNA polymerase α subunit that had been expressed in *E. coli* and to reconstitute enzymatically active RNA polymerase holoenzyme.

A. Methods:

Purification of M. tuberculosis α subunit: Recombinant α was produced in *E. coli* BL21(DE3) cells (Novagen) and grown at 37°C in LB medium containing 25 μg/ml/ kanamycin. Overnight cultures (100 ml) were diluted 10-fold and grown in LB medium containing 25 μg/ml kanamycin until A_{600} = 0.6 (approximately 75 min). Expression of α was induced for 3 hr with 4 mM IPTG. Purification of the α subunit was performd essentially as described by Tang, et. al, *Meth.Enzymol.* **273**:130, 1996. Briefly, cell pellets were harvested by centrifugation for 10 min At 4°C (3000 x g). The pellet was resuspended in 20 ml buffer B (6 M guanidine HCl, 10 mM Tris (pH 7.9), 500 mM NaCl, 5 mM imidazole) and lysed by sonication. Inclusion bodies were removed by centrifugation at 42,000 x g for 30 min at 4°C. The sample was then adsorbed onto Ni^{2+}-NTA (Qiagen) in buffer B. The sample was washed twice with 20 ml buffer B; washed twice with buffer B containing 30 mM imidazole; and eluted with 10 ml buffer B containing 500 mM imidazole. Adsorption, washes, and elution were performed with 1 min incubations at 4°C with gentle mixing.

Reconstitution of α with other M. tuberculosis subunits to form core/holo- RNA polymerase: Reconstitution of core/holo- enzyme was performed by combining 60, 300, and 600 μg of α, β, and β', respectively, followed slow removal of the denaturant by dialysis. The combined protein concentration was adjusted to 0.5 mg/ml with buffer B to prevent aggregation (Nobuyuki et al., *Meth.Enzymol* **273**:121, 1996) and dialysed against buffer E (50 mM Tris (pH 7.9), 200 mM KCl, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM EDTA, 5 mM β-mercaptoethanol, 20% (v/v) glycerol) overnight at 4°C. Following dialysis, the sample was activated by incubation for 45 min at 30°C and aggregates cleared by centrifugation at 10,000 x g for 10 min at 4°C. The sample was adsorbed onto Ni^{2+}-NTA in buffer F (50 mM Tris (pH

7.9), 0.5 mM EDTA, 5% (v/v) glycerol), and washed three times in buffer F containing 5 mM imidazole, and eluted in buffer F containing 150 mM imidazole. Adsorption and elution were performed by incubating for 45 min at 4°C and washes for 1 min. Each step was followed by centrifugation at 16,000 x g for 2 min at 4°C. Core/holo- enzyme was dialysed in buffer F (50 mM Tris (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 5% (v/v) glycerol) overnight at 4°C.

Purification of M. tuberculosis holoenzyme: Core polymerase and subassemblies were separated from holoenzyme by elution from a MonoQ column (Pharmacia) using a 0.2 - 0.5 M KCl gradient. Fractions containing holoenzyme were pooled and used the *in vitro* transcription assay.

Promoter construction: Recently, mycobacterium promoters have been cloned using a plasmid shuttle vector pSD7 (Das Gupta et al., *J. Bacteriol.* **175**:5186, 1993) and in a later study, the strength of these promoters was analyzed (Bashyam et al., *J. Bacteriol.* **178**:4847, 1996). Based on these studies two promoters, T125 and T101, identified as a weak and a strong promoter, respectively, were cloned into the pUC19 vector and used as templates for *M. tuberculosis* transcription asays.

Transcriptional activity: Holoenzyme was reconstituted by the addition of primary sigma factor MysA to the core polymerase and incubation at 30°C for 20 minutes. The transcription reaction was performed as described (Shorenstein et al., *J. Biol.Chem.* **248**:6170, 1973) except that 3 µg of template (pUC19, pMC116, or pMC117) was used per reaction containing a high salt transcription buffer (50 mM Tris (pH7.9), 10 $MgCl_2$, 200 mM KCl, 10 mM DTT, 0.1 mM EDTA, 1 mM K_2HPO_4 (pH7.5), 100 µg/ml BSA). 50 µl reactions were incubated for 30 minutes at 37°C. To precipitate the RNA transcripts and to stop the reaction, 100 µl of 10% TCA were added to the reaction. The TCA-precipitated RNA was

adsorbed onto UniFilter GF/C (Packard, Meriden, CT) double-thick glass fiber filtermats using a cell harvester (Packard, Meriden, CT). The wells of the microtiter plate and the filter were washed two times with 5% TCA and bound radioactivity was determined using a TopCount-HTS (Packard, Meriden, CT) scintillation counter.

B. Results:

Recombinant *M. tuberculosis* α subunit was overproduced in soluble form in *E. coli* cells transformed with plasmid pJH37, a derivative of pET26b, containing the entire *rpoA* coding region. *M. tuberculosis* α was expressed at high levels as a C-terminal hexahistidine-tagged fusion protein and purified to homogeneity by affinity chromatography. The majority of the 6xHis-tagged α subunit was located in the soluble fraction and was further purified using a Ni^{2+}-NTA column. Elution of the α subunit polypeptide was performed using 0.5 M imidazole, resulting in >90% recovery of the subunits with >95% purity as estimated by SDS-PAGE (Figure 5).

Core enzyme was reconstituted by combining crude preparations of the β and β' subunits with α polypeptide that contained a histidine tag, followed by removal of denaturant by dialysis to facilitate refolding. Holoenzyme was reconstituted by combining the core polymerase with purified primary σ (MysA) from *M. tuberculosis*. The core/ holoenzyme, containing a 6xHis tag on the α subunit, was purified from free subunits and subassemblies by batch elution from Ni^{2+}-NTA followed by elution from ion exchange chromatography (Figure 6).

To demonstrate that reconstituted *M. tuberculosis* RNA polymerase possesses transcriptional activity, *in vitro* transcription reactions were performed using MysA-containing

recombinant holoenzyme and templates containing two known MysA-dependent promoters (T101 and T125, which correspond to strong and weak mycobacterial promoters, respectively). The transcriptional activity of the reconstituted holoenzyme was more than two-fold higher when T101 was used as template as compared with T125 (Figure 7), suggesting that the rate of transcription was related to both the strength of the promoter and to the particular σ factor used.

These experiments demonstrated that *M. tuberculosis* α subunit produced according to the invention can be purified and reconstituted into enzymatically active RNA polymerase holoenzyme that exhibits specificity for *M. tuberculosis* promoters.

Example 3: <u>High Throughput Screens For Inhibitors of *M. Tuberculosis* RNA Polymerase and α Subunit</u>

High-throughput screens for anti-tuberculosis agents may be performed using either an *in vitro* or *in vivo* format. In either case, the ability of test compounds to inhibit *M. tuberculosis* RNA polymerase-driven transcription of *M. tuberculosis* promoters is tested. Typically, a strong promoter such as T101 is used for the test reactions. The sequence of the T101 promoter is:

T101:5'

AGCTTGCAGATCTAGCGATCGCAGCCGACGTGATACCTGACCGTTGTTGATA

GTGTCGGCGGCT-3'

a) In vitro screens:

The following procedure is used for cell-free high-throughput screening. A Tomtec Quadra 96-well pipetting station is used to add the reaction components to polypropylene 96-well dishes. 5 µl aliquots of test compounds dissolved in DMSO (or DMSO alone as a control) are added to wells. This is followed by 20 µl of the RNA polymerase mixture, which consists of: 10 mM DTT, 200 mM KCl, 10 mM Mg^{-2}, 1.5 µM bovine serum albumin, and 0.25 µg reconstituted RNA polymerase. After allowing the test compound to interact with the RNA polymerase, 25 µl of the DNA/NTP mixture is added, containing: 1 µg template DNA (see above), 4 µM [α-^{32}P]-UTP, and 400 µM each CTP, ATP, and GTP.

After incubation for 30 min at 25°C, the reaction is stopped by addition of 150 µl 10% trichloroacetic acid (TCA). After incubation at room temperature for 60 min, the TCA-precipitated RNA is adsorbed onto double-thick glass fiber filtermats using a Tomtec cell harvester. The wells of the microtiter plate and the filter are washed twice with 5% TCA and bound radioactivity is determined usinjg a Wallac microbeta 1450 scintillation counter.

Inhibitory activity due to the test compound is calculated according to the formula:

$$\% \text{ inhibition} = \frac{(cpm_{\text{positive control}} - cpm_{\text{sample}})}{cpm_{\text{positive control}}} \times 100$$

where $cpm_{\text{positive control}}$ represents the average of the cpm in wells that received DMSO alone, and cpm_{sample} represents the cpm in the well that received test compound. Compounds that cause at least 50% inhibition are scored as positive "hits" in this assay.

As an additional control, rifampicin is used at a concentration of 30 nM, which results in a 50-75% inhibition of transcription in this assay.

b) In vivo screen:

M. tuberculosis RNA polymerase subunits (α, β, β', and a particular σ subunit) are expressed in *E. coli* under the control of regulatable promoters by transforming *E. coli* with appropriate plasmids. If the σ^A subunit is expressed, a DNA sequence comprising the T101 promoter is also introduced into the cells to serve as a template for *M. tuberculosis*-specific transcription.

In one embodiment, the T101 promoter sequence is linked to a DNA sequence encoding the xylE gene product, catechol 2, 3-dioxygenase (CDO). When expressed in the *E. coli* cell, CDO converts catechol to 2-hydroxymuconic semialdehyde, which has a bright yellow color (having an absorbance maximum at 375 nm) that is easily detected in whole cells or in crude extracts. The substrate for this enzyme is a small aromatic molecule that easily enters the bacterial cytoplasm and does not adversely affect cell viability.

In a high-throughput format, aliquots of bacterial cultures are incubated in the absence or presence of test compounds, and CDO activity is monitored by measuring absorbance at 375 nm following addition of catechol.

c) Specificity:

Compounds that score as positive in either the *in vitro* or *in vivo* assays described above are then tested for their effect on human RNA polymerase II. Those compounds which do not significantly inhibit human RNA polymerase II will be further developed as potential anti-tuberculosis agents.

Claims:

1. An isolated, purified DNA encoding *M. tuberculosis* RNA polymerase α subunit.

2. A DNA as defined in claim 1. wherein said DNA has a sequence selected from the group consisting of the sequence shown in Figure 3, sequence-conservative variants thereof, and function-conservative variants thereof.

3. A DNA vector comprising the nucleic acid sequence of claim 2 operably linked to a transcription regulatory element.

4. A cell comprising a DNA vector as defined in claim 3, wherein said cell is selected from the group consisting of bacterial, fungal, plant, insect, and mammalian cells.

5. A cell as defined in claim 4, wherein said cell is a bacterial cell.

6. An isolated purified polypeptide comprising a polypeptide encoded by a DNA as defined in claim 2.

7. A method for high-throughput screening to detect inhibitors of *M. tuberculosis* RNA polymerase, said method comprising:

a) providing a mixture comprising

(i) purified *M. tuberculosis* RNA polymerase,

(ii) a DNA template encoding a promoter sequence that is recognized by *M. tuberculosis* RNA polymerase;

b) incubating said mixture in the presence of test compounds to form test samples, and in the absence of test compounds to form control samples, wherein said incubating is performed under conditions that result in RNA synthesis in the control samples;

c) measuring RNA synthesis directed by said *M. tuberculosis*-recognized promoter in said test and control samples; and

d) comparing the RNA synthesis detected in step (c) between said test and control samples;

wherein an RNA polymerase inhibitor is a test compound that causes a reduction in RNA synthesis measured in said test sample relative to RNA synthesis measured in said control sample.

8. A method as defined in claim 7, wherein said purified *M. tuberculosis* RNA polymerase comprises recombinant subunits which are reconstituted to form an enzymatically active holoenzyme.

9. A method for high-throughput screening to detect inhibitors of *M. tuberculosis* RNA polymerase, said method comprising:

a) providing a non-mycobacterial bacterial strain that

(i) has been transformed with a DNA template encoding a promoter sequence that is recognized by *M. tuberculosis* RNA polymerase, and

(ii) expresses enzymatically active *M. tuberculosis* RNA polymerase, wherein said polymerase comprises α, β, β', and one of several σ subunits;

b) incubating the bacterial strain of (a) in the presence of test compounds to form test samples, and in the absence of test compounds to form control samples;

c) measuring RNA synthesis directed by said *M. tuberculosis*-recognized promoter in the test and control samples; and

d) comparing the RNA synthesis detected in step (c) between the test and control samples;

wherein an RNA polymerase inhibitor is a test compound that causes a reduction in RNA synthesis measured in said test sample relative to RNA synthesis measured in said control sample.

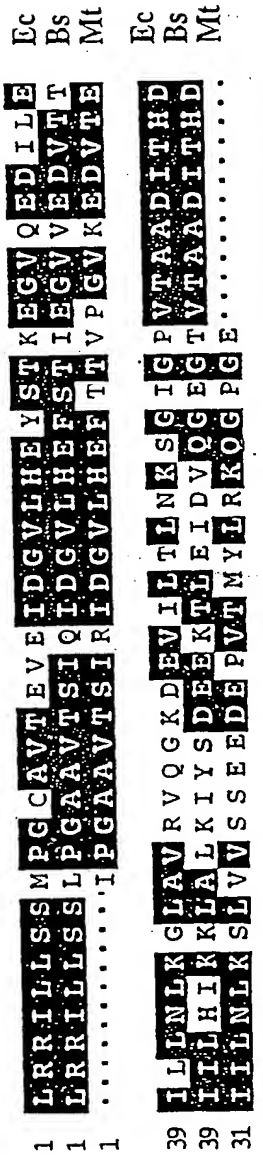

Fig. 1

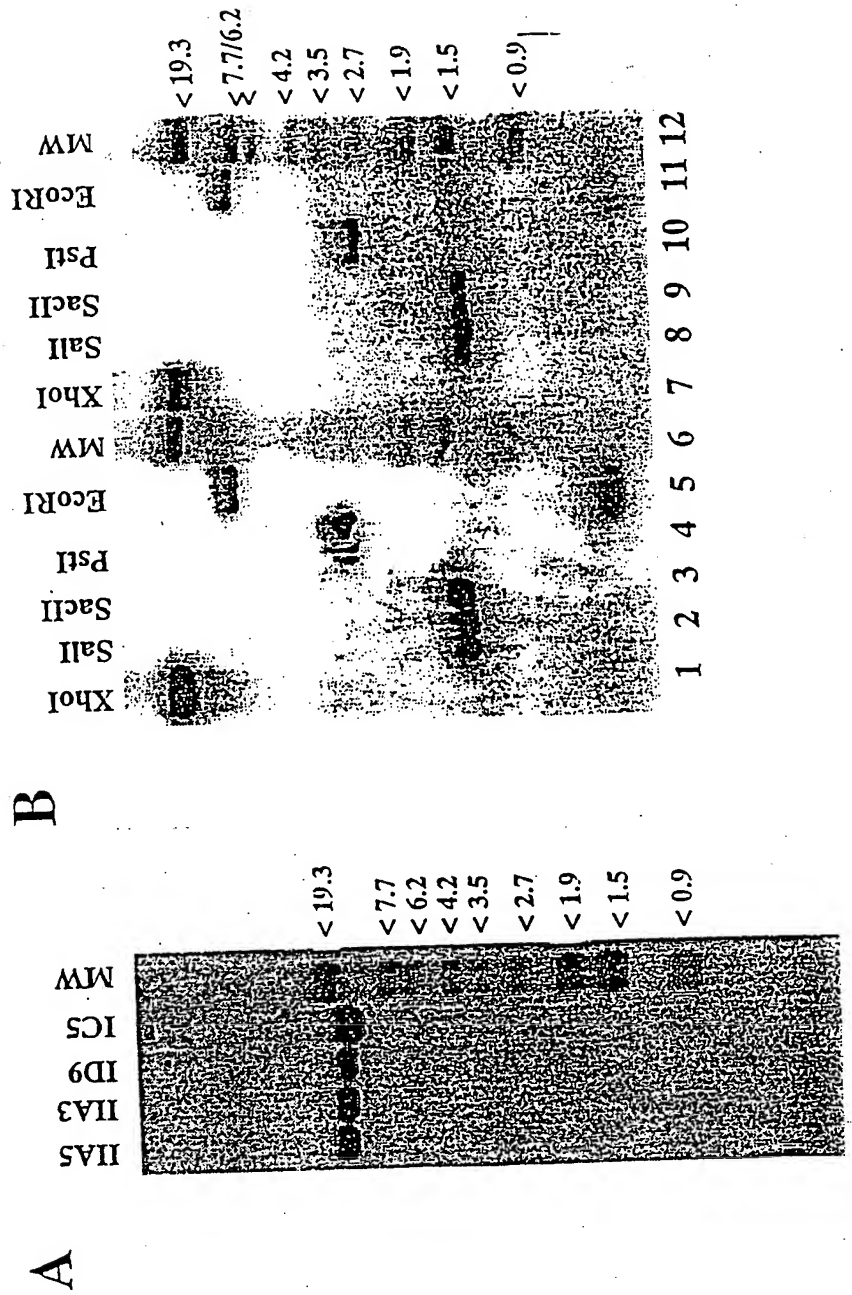

Fig. 2

```
1/1                                                                 31/11
CGA GTA CCC CCA CCT TCG GGG GCG CCG CCC CCG AGT GCC CCC ACA GAC GTC ATA TGG CGG
R   V   P   P   P   S   G   A   P   P   P   S   A   P   T   D   V   I   W   R
61/21     RBS                                                       91/31
ACG TCG AAA GGA AGA AGA AAC ACC ATG CTG ATC TCA CAG CGC CCC ACC CTG TCC GAG GAC
T   S   K   G   R   R   N   T   M   L   I   S   Q   R   P   T   L   S   E   D
121/41                                                              151/51
GTC CTC ACC GAC AAC CGA TCC CAG TTC GTG ATC GAA CCG CTG GAG CCG GGA TTC GGC TAC
V   L   T   D   N   R   S   Q   F   V   I   E   P   L   E   P   G   F   G   Y
181/61                                                              211/71
ACC CTG GGC AAT TCG CTG CGT CGC ACC CTG CTG TCG TCG ATT CCC GGA GCG GCC GTC ACC
T   L   G   N   S   L   R   R   T   L   L   S   S   I   P   G   A   A   V   T
241/81                                                              271/91
AGC ATT CGC ATC GAT GGT GTA CTG CAC GAA TTC ACC ACG GTG CCC GGG GTC AAA GAA GAT
S   I   R   I   D   G   V   L   H   E   F   T   T   V   P   G   V   K   E   D
301/101                                                             331/111
GTC ACC GAG ATC ATC CTG AAT CTC AAG AGC CTG GTG GTG TCC TCG GAG GAG GAC GAG CCG
V   T   E   I   I   L   N   L   K   S   L   V   V   S   S   E   E   D   E   P
361/121                                                             391/131
GTC ACC ATG TAC CTA CGC AAG CAG GGT CCG GGT GAG GTT ACC GCC GGC GAC ATC GTG CCG
V   T   M   Y   L   R   K   Q   G   P   G   E   V   T   A   G   D   I   V   P
421/141                                                             451/151
CCG GCC GGC GTC ACC GTG CAC AAC CCC GGC ATG CAC ATC GCC ACG CTG AAC GAT AAG GGC
P   A   G   V   T   V   H   N   P   G   M   H   I   A   T   L   N   D   K   G
481/161                                                             511/171
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K   L   E   V   E   L   V   V   E   R   G   R   G   Y   V   P   A   V   Q   N
541/181                                                             571/191
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R   A   S   G   A   E   I   G   R   I   P   V   D   S   I   Y   S   P   V   L
601/201                                                             631/211
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661/221                                                             691/231
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I   L   D   V   E   T   K   N   S   I   S   P   R   D   A   L   A   S   A   G
721/241                                                             751/251
AAG ACG CTG GTC GAG TTG TTC GGC CTG GCA CGG GAA CTC AAC GTC GAG GCC GAA GGC ATC
K   T   L   V   E   L   F   G   L   A   R   E   L   N   V   E   A   E   G   I
781/261                                                             811/271
GAG ATC GGG CCG TCG CCG GCC GAG GCC GAT CAC ATT GCG TCA TTC GCC CTG CCG ATC GAC
E   I   G   P   S   P   A   E   A   D   H   I   A   S   F   A   L   P   I   D
841/281                                                             871/291
GAC CTG GAT CTG ACG GTG CGG TCC TAC AAC TGC CTC AAG CGC GAG GGG GTG CAC ACC GTG
D   L   D   L   T   V   R   S   Y   N   C   L   K   R   E   G   V   H   T   V
901/301                                                             931/311
GGC GAA CTG GTG GCG CGC ACC GAA TCC GAC CTG CTT GAC ATC CGC AAC TTC GGT CAG AAG
G   E   L   V   A   R   T   E   S   D   L   L   D   I   R   N   F   G   Q   K
961/321                                                             991/331
TCC ATC GAC GAG GTG AAG ATC AAG CTG CAC CAG CTG GGC CTG TCA CTC AAG GAC AGC CCG
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1021/341                                                            1051/351
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P   S   F   D   P   S   E   V   A   G   Y   D   V   A   T   G   T   W   S   T
1081/361                                                            1111/371
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E   G   A   Y   D   E   Q   D   Y   A   E   T   E   Q   L   *   T   A   S   N
1141/381
CCA GAC AGG AGC GTC AGC TAT GCC CAA GCC
P   D   R   S   V   S   Y   A   Q   A
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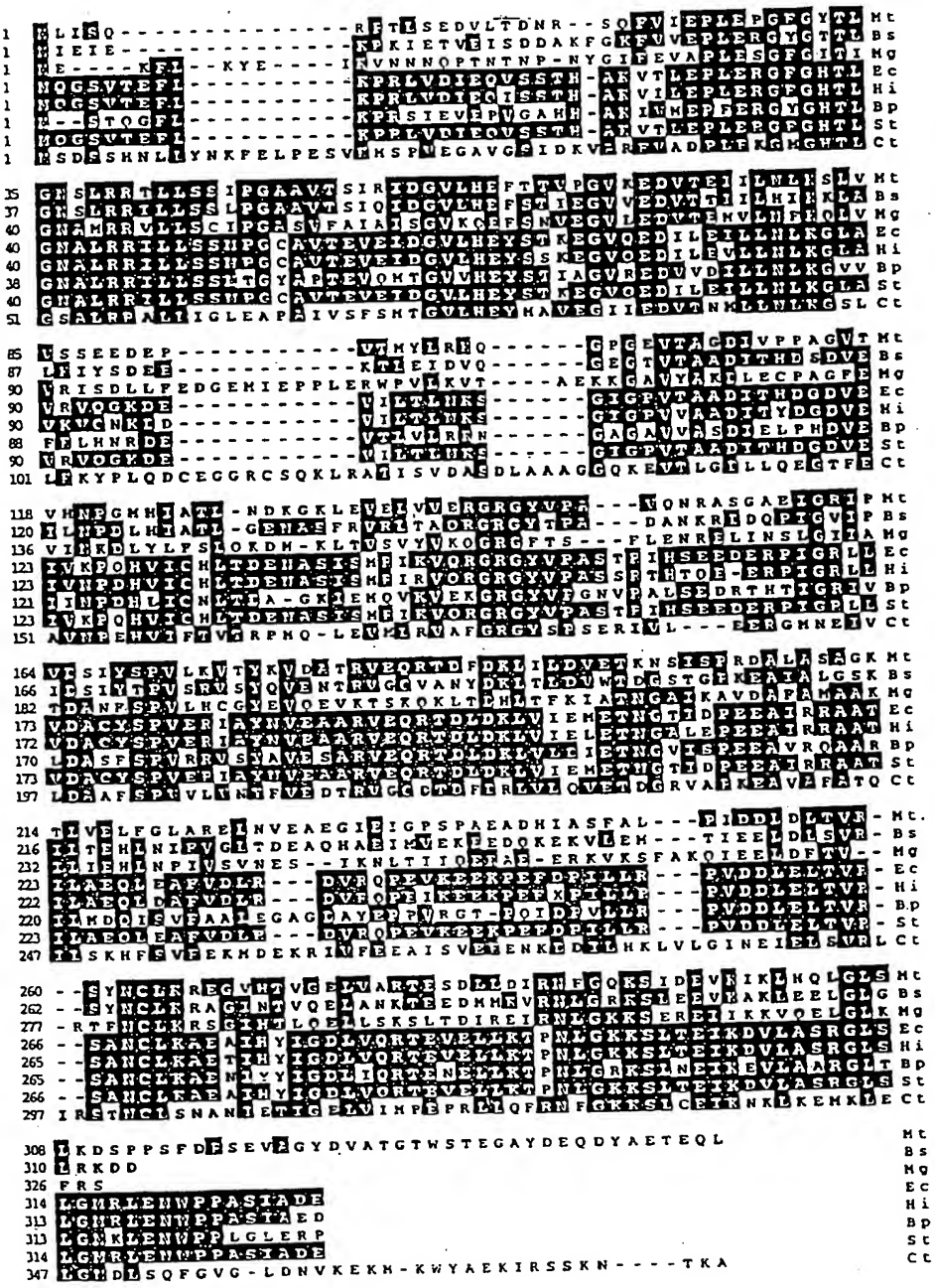

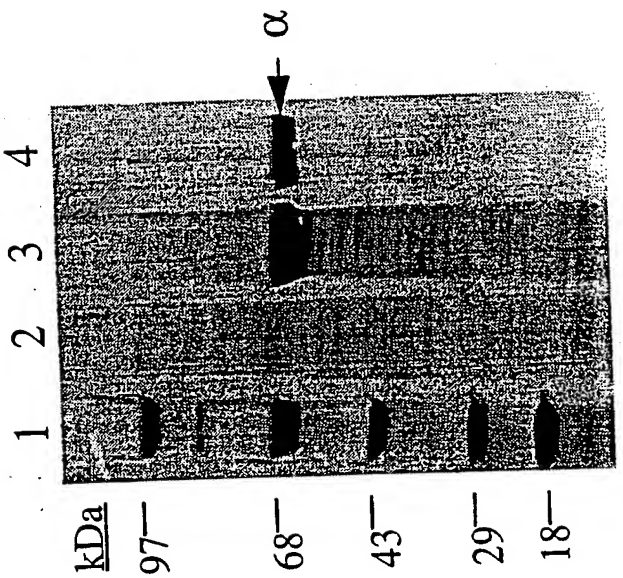

Figure 5.

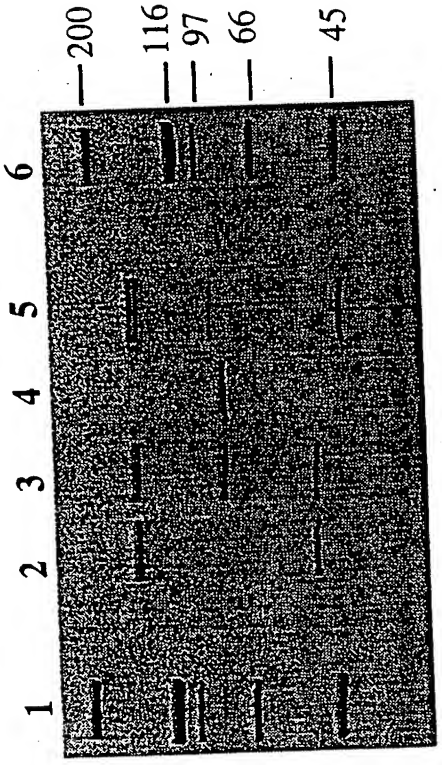

Figure 6.

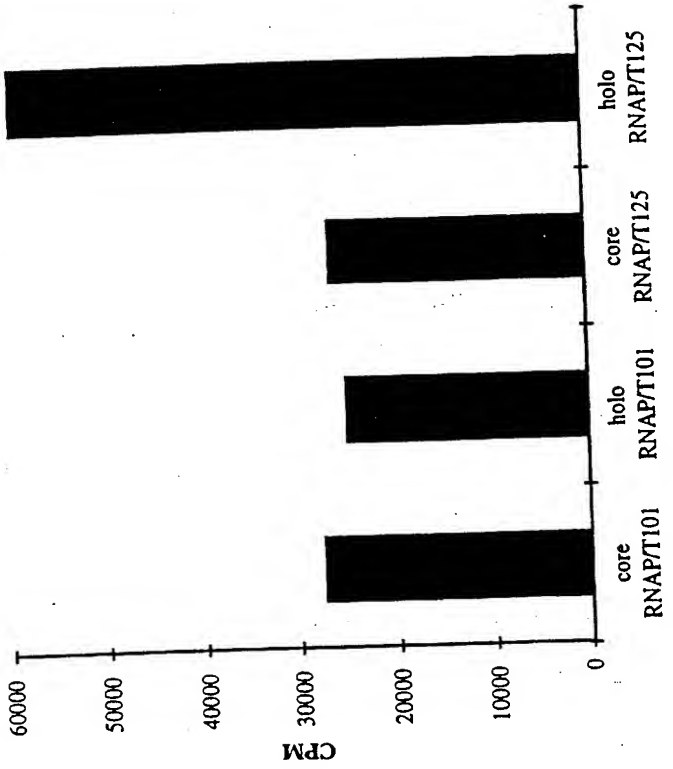
CPM
60000
50000
40000
30000
20000
10000
0
core RNAP/T101
holo RNAP/T101
core RNAP/T125
holo RNAP/T125

Figure 7.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/22216

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 9/12, 15/54
US CL :435/194, 15, 325, 419, 252.3, 320.1; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/194, 15, 325, 419, 252.3, 320.1; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG
search terms: RNA polymerase, alpha subunit, Mycobacterium tuberculosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HARSHEY et al. Purification and properties of DNA-dependent RNA polymerase from *Mycobacterium tuberculosis* $H_{37}R_v$. Biochimica et Biophysica Acta. 1976, Vol. 432, No. 1, pages 49-59, see entire document.	1-9
A	MILLER et al. The *rpoB* gene of *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. April 1994, Vol. 38, No. 4, pages 805-811, see entire document.	1-9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 JANUARY 1998	Date of mailing of the international search report 23 FEB 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KEITH D. HENDRICKS Telephone No. (703) 308-0196

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VERSION*

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(54) Title: M. TUBERCULOSIS RNA POLYMERASE ALPHA SUBUNIT

(57) Abstract

The present invention provides isolated nucleic acids encoding RNA polymerase alpha subunit from *M. tuberculosis*, vectors comprising the nucleic acids, cells comprising the vectors, and methods for producing *M. tuberculosis* alpha subunit. The invention also provides *in vitro* and *in vivo* methods for high–throughput screening to identify inhibitors of *M. tuberculosis* RNA polymerase.

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M. TUBERCULOSIS RNA POLYMERASE ALPHA SUBUNIT

Field of the Invention

The present invention relates to novel nucleic acids encoding RNA polymerase alpha subunit from *M. tuberculosis* and methods for use thereof.

Background of the Invention

The intracellular pathogen *Mycobacterium tuberculosis* is the causative agent of tuberculosis in humans and is responsible for millions of deaths worldwide each year (Bloom et al. *Science* 257:1055, 1992). The emergence of multidrug resistant forms of tuberculosis has mandated the development of new antibiotics effective against refractory *M. tuberculosis* strains. Novel drugs which, like rifampin (Vall-Spinosa et al., *N. Eng. J. Med.* 283: 616, 1970), may be capable of inhibiting the prokaryotic transciptional machinery, could contribute significantly to the development of therapies to combat *M. tuberculosis*.

The RNA polymerase of eubacteria consists of four subunits (α, β, β', and σ) and exists in two major forms: core enzyme (α_2, β, β') and holoenzyme (α_2, β, β' plus one of

several σ subunits) (Chamberlin, in *RNA Polymerase*, Losick et al., eds., Cold Spring Harbor, 1976, pp. 17-67). The α subunit contains determinants for protein-protein interactions with transcription activators and protein-DNA interaction with upstream elements (Ishihama, *Mol.Microbiol.* 6:3283, 1992; Russo et al., *J. Biol.Chem.* 267:14515, 1992; Ebright et al., *Curr.Opin.Genet.Dev.* 5:197, 1995). The amino terminal domain of α is also required for assembly of the multisubunit core RNA polymerase (Ishihama, *Adv. Biophys.* 14:1, 1981). The β subunit is essential for transcript initiation and elongation, which β' apparently functions in binding of the core enzyme to template DNA (Yura et al., *Ann.Rev.Genet.* 13:59, 1979). Core RNA polymerase is capable of RNA synthesis; however, σ is required for specific initiation of transcripts at promoter sequences (Gross et al., in *Transcriptional Regulation*, Cold Spring Harbor, 1992, pp. 129-176).

Discovery of inhibitors of *M. tuberculosis* RNA polymerase is hampered by a lack of information concerning components of the *M. tuberculosis* transcriptional apparatus, difficulties in obtaining sufficient yields of active enzymes for biochemical studies, and biosafety concerns. Establishment of an *in vitro* transcription system employing purified and reconstituted RNA polymerase would greatly advance efforts to identify new therapeutic agents active against tuberculosis.

Accordingly, there is a need in the art for compositions and methods utilizing cloned genes and purified proteins derived from *M. tuberculosis* RNA polymerase.

Summary of the Invention

The present invention is based on the isolation and characterization of DNA encoding the α subunit of RNA polymerase derived from *M. tuberculosis*. In one aspect, the invention provides a purified, isolated nucleic acid having the sequence shown in Figure 3. The invention also encompasses sequence-conservative and function-conservative variants of this sequence. The invention also provides vectors comprising these sequences, and cells comprising the vectors.

In another aspect, the present invention provides a purified, isolated polypeptide encoded by the nucleic acid sequence shown in Figure 3, as well as function-conservative variants thereof. In one embodiment, the invention provides a purified α subunit further comprising a hexahistidine tag. The invention also provides purified, reconstituted core- and holoenzyme comprising the α subunit.

In yet another aspect, the invention provides *in vitro* methods for high-throughput screening to detect inhibitors of *M. tuberculosis* RNA polymerase. In one embodiment, the methods comprise:

a) providing a mixture comprising

(i) purified *M. tuberculosis* RNA polymerase and

(ii) a DNA template encoding a promoter sequence that is recognized by *M. tuberculosis* RNA polymerase;

b) incubating the mixture in the presence of test compounds to form test samples, and in the absence of test compounds to form control samples, under conditions that result in RNA synthesis in the control samples;

c) measuring RNA synthesis directed by said *M. tuberculosis*-recognized promoter in the test and control samples; and

d) comparing the RNA synthesis detected in step (c) between the test and control samples. According to the invention, an RNA polymerase inhibitor is a test compound that causes a reduction in RNA synthesis directed by the *M. tuberculosis*-recognized promoter measured in the test sample relative to RNA synthesis measured in the control sample. In a preferred embodiment, the purified RNA polymerase used in practicing this method comprises recombinant subunits which are reconstituted to form an enzymatically active holoenzyme. In another embodiment, the ability of test compounds to bind to purified α subunit is monitored.

In yet another aspect, the invention provides *in vivo* methods for high-throughput screening to detect inhibitors of *M. tuberculosis* RNA polymerase. The methods are carried out by the steps of:

a) providing a non-mycobacterial bacterial strain, preferably *E. coli*, that

(i) has been transformed with a DNA template encoding a promoter sequence that is recognized by *M. tuberculosis* RNA polymerase, and

(ii) expresses enzymatically active *M. tuberculosis* RNA polymerase (e.g., α_2, β, β' plus one of several σ subunits);

b) incubating the bacterial strain of (a) in the presence of test compounds to form test samples, and in the absence of test compounds to form control samples;

c) measuring RNA synthesis directed by said *M. tuberculosis*-recognized promoter in the test and control samples; and

d) comparing the RNA synthesis directed by said *M. tuberculosis*-recognized promoter detected in step (c) between the test and control samples. According to the invention, an RNA polymerase inhibitor is a test compound that causes a reduction in RNA synthesis measured in the test sample relative to RNA synthesis measured in the control sample.

These and other aspects of the present invention will be apparent to those of ordinary skill in the art in light of the present specification and appended claims.

Brief Description of the Drawings

Figure 1 is a graphic illustration comparing the deduced amino acid sequence of the *M. tuberculosis rpoA* gene fragment and corresponding regions of α subunits of *B. subtilis* and *E. coli*. Mt, *M. tuberculosis*; Bs, *B. subtilis*; Ec, *E. coli*. Dashed lines denote conserved segments of *B. subtilis* and *E. coli* proteins for which oligonucleotides were designed.

Figure 2A is a photographic illustration of a Southern blot analysis of *rpoA* hybridizing cosmid clones. Four different positive clones (designated IIA5, IIA3, ID9, and IC5) isolated from an *M. tuberculosis* cosmid library were digested with *Not*I and hybridized with an *rpoA* gene probe. Sizes of DNA markers (λ EcoT141, Amersham) are indicated in kb.

Figure 2B is a photographic illustration of a Southern blot analysis of *rpoA* hybridizing cosmid clones, showing structural conservation of genomic and cosmid-borne *rpoA* sequences. Purified cosmid IC5 DNA (lanes 1-5) or *M. tuberculosis* H37Rv genomic DNA (lanes 7-11) were digested with the indicated restriction enzymes and hybridized with an *rpoA* probe. Sizes of DNA markers (λ EcoT141, Amersham) are indicated in kb.

Figure 3 shows the nucleotide and deduced amino acid sequences of the *M. tuberculosis* H37Rv *rpoA* gene. RBS denotes a potential ribosome binding site. The putative start and stop codons of the *rpoA* gene product are at 85-87 and 1126-1128, respectively.

Figure 4 is a graphic illustration comparing the inferred amino acid sequence of the *M. tuberculosis* H37Rv α subunit with sequences of α subunits from other bacteria. *B. subtilis* (Bs), *M. genitalium* (Mg), *E. coli* (Ec), *H. influenza* (Hi), *B. pertusis* (Bp), *S. typhimurium* (St), and *C. trachomitis* (Ct). Shading indicates identical amino acid residues. Amino acid sequence alignments were performed using MegAlign (DNAStar).

Figure 5 is a photographic illustration of an SDS-polyacrylamide gel electropherogram depicting the expression and purification of *M. tuberculosis* α subunit in *E. coli*. Lane 1 shows molecular mass markers (kDa); lanes 2 and 3 show total proteins from uninduced and IPTG-induced cultures, respectively, of *E. coli* harboring pJH37; and lane 4 shows purified *M. tuberculosis* α subunit (approximately 38 kDa), which is indicated by an arrow.

Figure 6 is a photographic illustration of an SDS-polyacrylamide gel electropherogram showing the reconstitution and purification of core- and holo- RNA polymerase from *M. tuberculosis*. Lanes 1 and 6 show molecular mass markers (kDa); lane 2 shows α-histidine-containing core enzyme; lane 3 shows a histidine-containing holoenzyme; lane 4 shows N-terminal histidine-tagged primary sigma; and lane 5 shows purified *E. coli* holoenzyme.

Figure 7 is a graphic illustration of *in vitro* transcription reactions employing reconstituted purified *M. tuberculosis* RNA polymerase holoenzyme and T101 and T125 promoter-containing DNA templates.

Detailed Description of the Invention

All patent applications, patents and literature references cited herein are hereby incorporated by reference in their entirety. In the case of inconsistencies the present disclosure will prevail.

The present invention is based on the isolation of a fragment of the *M. tuberculosis rpoA* gene, encoding the α subunit of RNA polymerase. As described in Example 1 below, the fragment was isolated by the polymerase chain reaction (PCR) using primers directed against regions of α that are highly conserved among Gram-positive and Gram-negative bacteria. The amplified DNA was utilized as a hybridization probe to recover the entire *rpoA* gene from a cosmid library of genomic DNA from virulent *M. tuberculosis* strain H37RV. Nucleotide sequencing indicated that the 1044 bp *M. tuberculosis rpoA* open reading frame (ORF) encodes a protein of 347 amino acids which shows significant structural similarity to the α subunits of diverse bacterial species with greatest identity to the *B. subtilis* α protein (Figure 1).

Though structurally conserved throughout most of its length, the inferred amino acid sequence of the *M. tuberculosis* α subunit diverged from that of *B. subtilis* and other bacteria at its extreme C-terminus. Interestingly, studies on *E. coli* RNA polymerase subunits have indicated that the C-terminal 94 residues of α function in interactions with certain transcriptional regulatory proteins, while the N-terminal domain may play a more highly conserved role in facilitating the core assembly (Ishihama, *Mol.Microbiol.* 6:3283, 1992; Russo et al., *J.Biol.Chem.* 267:14515, 1992).

The *B. subtilis rpoA* gene is part of a large operon that is cotranscribed with genes for initiation factor 1 and ribosomal proteins B, S13, S11, and L17; and, unlike the pattern of gene organization seen in *E. coli*, the *B. subtilis rpoA* gene cluster is genetically linked to the *rpoB-C* operon encoding RNA polymerase components β and β' (Boylan et al., *J. Bacteriol.* 171:2553, 1992).

In practicing the present invention, many techniques in molecular biology, microbiology, recombinant DNA, and protein biochemistry such as these explained fully in, for example, Sambrook *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Second

Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; *DNA Cloning: A Practical Approach*, Volumes I and II, 1985 (D.N. Glover ed.); *Oligonucleotide Synthesis*, 1984, (M.L. Gait ed.); *Transcription and Translation*, 1984 (Hames and Higgins eds.); *A Practical Guide to Molecular Cloning*; the series, *Methods in Enzymology* (Academic Press, Inc.); and *Protein Purification: Principles and Practice*, Second Edition (Springer-Verlag, N.Y.), may be used.

The present invention encompasses nucleic acid sequences encoding the α subunit of *M. tuberculosis* RNA polymerase, enzymatically active fragments derived therefrom, and related sequences. As used herein, a nucleic acid that is "derived from" a sequence refers to a nucleic acid sequence that corresponds to a region of the sequence, sequences that are homologous or complementary to the sequence, and "sequence-conservative variants" and "function-conservative variants". Sequence-conservative variants are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position. Function-conservative variants are those in which a given amino acid residue in the α subunit has been changed without altering the overall conformation and function of the polypeptide, including, but not limited to, replacement of an amino acid with one having similar physico-chemical properties (such as, for example, acidic, basic, hydrophobic, and the like). Fragments of the α subunit that retain enzymatic activity can be identified according to the methods described herein, such as e.g., expression in *E. coli* followed by enzymatic assay of the cell extract or by purification and reconstitution of the purified polypeptide into an enzymatically active holoenzyme.

The nucleic acids of the present invention include purine- and pyrimidine-containing polymers of any length, either polyribonucleotides or polydeoxyribonucleotides or mixed polyribo-polydeoxyribo nucleotides. This includes single- and double-stranded molecules, i.e., DNA-DNA, DNA-RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone. This also includes nucleic acids containing modified bases. The nucleic acids may be isolated directly from cells. Alternatively, PCR can be used to produce the nucleic acids of the invention, using either chemically synthesized strands or genomic material as templates. Primers used for PCR can be synthesized using the sequence information provided herein and can further be designed to introduce appropriate new restriction sites, if desirable, to facilitate incorporation into a given vector for recombinant expression.

The nucleic acids of the present invention may be flanked by natural *M. tuberculosis* regulatory sequences, or may be associated with heterologous sequences, including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5'- and 3'- noncoding regions, and the like. The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, and internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.). Nucleic acids may contain one or more additional covalently linked moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (e.g., acridine, psoralen, etc.), chelators (e.g., metals, radioactive metals, iron, oxidative metals, etc.), and alkylators. The nucleic acid may be derivatized by formation of a methyl or ethyl phosphotriester or an alkyl phosphoramidate linkage. Furthermore, the nucleic acid sequences of the present invention may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

The invention also provides nucleic acid vectors comprising the disclosed α subunit sequences or derivatives or fragments thereof. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. Non-limiting examples include pKK plasmids (Clontech), pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), or pRSET or pREP (Invitrogen, San Diego, CA), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, e.g. antibiotic resistance, and one or more expression cassettes. Suitable host cells may be transformed/transfected/infected as appropriate by any suitable method including electroporation, $CaCl_2$ mediated DNA uptake, fungal infection, microinjection, microprojectile, or other established methods.

Appropriate host cells include bacteria, archebacteria, fungi, especially yeast, and plant and animal cells, especially mammalian cells. Of particular interest are *E. coli*,

B. subtilis, Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Schizosaccharomyces pombi, SF9 cells, C129 cells, 293 cells, *Neurospora*, and CHO cells, COS cells, HeLa cells, and immortalized mammalian myeloid and lymphoid cell lines. Preferred replication systems include M13, ColE1, SV40, baculovirus, lambda, adenovirus, and the like. A large number of transcription initiation and termination regulatory regions have been isolated and shown to be effective in the transcription and translation of heterologous proteins in the various hosts. Examples of these regions, methods of isolation, manner of manipulation, etc. are known in the art. Under appropriate expression conditions, host cells can be used as a source of recombinantly produced Mycobacterial-derived peptides and polypeptides.

Advantageously, vectors may also include a transcription regulatory element (i.e., a promoter) operably linked to the α subunit portion. The promoter may optionally contain operator portions and/or ribosome binding sites. Non-limiting examples of bacterial promoters compatible with *E. coli* include: *trc* promoter, β-lactamase (penicillinase) promoter; lactose promoter; tryptophan (trp) promoter; arabinose BAD operon promoter; lambda-derived Pl promoter and N gene ribosome binding site; and the hybrid tac promoter derived from sequences of the trp and lac UV5 promoters. Non-limiting examples of yeast promoters include 3-phosphoglycerate kinase promoter, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, galactokinase (GALI) promoter, galactoepimerase promoter, and alcohol dehydrogenase (ADH) promoter. Suitable promoters for mammalian cells include without limitation viral promoters such as that from Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus (ADV), and bovine papilloma virus (BPV). Mammalian cells may also require terminator sequences and poly A addition sequences, and enhancer sequences which increase expression may also be included. Sequences which cause amplification of the gene may also be desirable. Furthermore, sequences that facilitate secretion of the recombinant product from cells, including, but not limited to, bacteria, yeast, and animal cells, such as secretory signal sequences and/or prohormone pro region sequences, may also be included.

Nucleic acids encoding wild-type or variant α subunit polypeptides may also be introduced into cells by recombination events. For example, such a sequence can be introduced into a cell, and thereby effect homologous recombination at the site of an endogenous gene or a sequence with substantial identity to the gene. Other recombination-

based methods, such as non-homologous recombinations or deletion of endogenous genes by homologous recombination, may also be used.

α subunit-derived polypeptides according to the present invention, including function-conservative variants, may be isolated from wild-type or mutant *M. tuberculosis* cells, or from heterologous organisms or cells (including, but not limited to, bacteria, fungi, insect, plant, and mammalian cells) into which an α subunit-derived protein-coding sequence has been introduced and expressed. Furthermore, the polypeptides may be part of recombinant fusion proteins (see, e.g., Example 2 below). Alternatively, polypeptides may be chemically synthesized by commercially available automated procedures, including, without limitation, exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis.

"Purification" of an α subunit polypeptide refers to the isolation of the polypeptide in a form that allows its enzymatic activity to be measured without interference by other components of the cell in which the polypeptide is expressed. Methods for polypeptide purification are well-known in the art, including, without limitation, preparative disc-gel electrophoresis, isoelectric focusing, HPLC, reversed-phase HPLC, gel filtration, ion exchange and partition chromatography, and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine sequence. The polypeptide can then be purified from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against the α subunit or against peptides derived therefrom can be used as purification reagents. Other purification methods are possible.

The isolated polypeptides may be modified by, for example, phosphorylation, sulfation, acylation, or other protein modifications. They may also be modified with a label capable of providing a detectable signal, either directly or indirectly, including, but not limited to, radioisotopes and fluorescent compounds.

Screening Methods to Identify Anti-tuberculosis Agents

The methods and compositions of the present invention can be used to identify compounds that inhibit the function of *M. tuberculosis* RNA polymerase and thus are useful as anti-tuberculosis agents. This is achieved by providing enzymatically active recombinant α subunit according to the present invention, in combination with other

components of RNA polymerase, in a context in which the inhibitory effects of test compounds can be measured. Alternatively, the ability of test compounds to bind to purified α subunit is monitored, using methods well-known in the art. Test compounds identified using the methods of the invention encompass those that interfere with the enzymatic activity of RNA polymerase and/or disrupt the interaction of the α subunit with transcriptional activators. Thus, the compounds may function as general or gene-specific transcriptional inhibitors.

In a preferred embodiment, recombinant *M. tuberculosis* RNA polymerase subunits (α, β, β' plus one of several σ subunits) are purified in milligram quantities from *E. coli* cultures by affinity methods utilizing a hexahistidine tagged α subunit. Enzymatically active holoenzyme is reconstituted using these components (see, e.g., Example 2 below). The active polymerase is then incubated in the presence of test compounds to form test mixtures, and in the absence of test compounds to form control mixtures. *In vitro* transcription is then carried out using a DNA template containing appropriate promoter sequences (see, e.g., Example 2 below).

In another embodiment, *M. tuberculosis* RNA polymerase subunits (α_2, β, β' plus one of several σ subunits) are co-expressed in *E. coli* or another surrogate bacterial cell, in conjunction with an appropriate promoter functionally linked to a reporter gene. The ability of test compounds to differentially inhibit *M. tuberculosis* RNA polymerase is then assessed by measuring the amount and/or activity of the reporter gene product.

M. tuberculosis promoters useful in practicing the invention include without limitation: P1 or P2 hsp 60 promoters (Stover et al., *Nature* **351**:456, 1991); cpn-60 promoter (Kong et al., *Proc.Natl.Acad.Sci.USA* **90**:2608, 1993); 85A antigen promoter (Kremer, *J. Bacteriol.* **177**:642, 1995); PAN promoter (Murray et al., *Mol. Microbiol.* **6**:3331, 1992); 16S RNA promoter (Ji et al., *Microbiol.* **140**: 2829, 1994); and askβ promoter (Cirillo et al., *Mol. Microbiol.* **11**:629, 1994). Useful reporter genes include without limitation xylE (Curcic et al., *Mol. Microbiol* **13**:1057, 1994); CAT (Das Gupta et al., *J. Bacteriol.* **175**:5186, 1993); luciferase (Cooksey et al., *Antimicrob.Agts.Chemother.* **37**:1348, 1993); green fluorescent protein (Dhadayuthap et al., *Mol. Microbiol.* **17**:901, 1995); and lacZ (Silhavy et al., *Microbiol.Rev.* **49**:398, 1985).

It will be understood that the present invention encompasses *M. tuberculosis* RNA polymerases containing any appropriate σ factor, which is used in conjunction with a particular promoter that is recognized by RNA polymerase containing that σ factor.

Non-limiting examples of useful σ factors include σ^A, σ^B, and σ^F (Doushkan et al., 1995; DeMaio et al., *Gene* 165:67, 1995). The invention also encompasses the identification of additional promoters that are recognized by a particular σ subunit. This is achieved by providing a library of random *M. tuberculosis* gene fragments cloned upstream of an appropriate reporter gene (see above). The library is transformed into *M. tuberculosis* or *M. smegmatis* and reporter gene expression is measured. Alternatively, the library is transformed into another bacterial cell, such as, e.g., *E. coli*, which expresses *M. tuberculosis* RNA polymerase core subunits as well as a σ subunit of interest and cognate promoters that drive reporter gene expression. In yet another embodiment, expression of an *M. tuberculosis* σ factor confers new recognition properties on *E. coli* RNA polymerase and permits isolation of promoters utilized specifically by a particular *M. tuberculosis* σ subunit.

Preferably, both *in vitro* and *in vivo* screening methods to detect *M. tuberculosis*-specific RNA polymerase inhibitors are adapted to a high-throughput format, allowing a multiplicity of compounds to be tested in a single assay. Such inhibitory compounds may be found in, for example, natural product libraries, fermentation libraries (encompassing plants and microorganisms), combinatorial libraries, compound files, and synthetic compound libraries. For example, synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich Chemical Company, Inc. (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from, for example, Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle et al., *TibTech* 14:60, 1996). preferably using automated equipment, to allow for the simultaneous screening of a multiplicity of test compounds.

Useful anti-tuberculosis compounds are identified as those test compounds that decrease tuberculosis-specific transcription. Once a compound has been identified by the methods of the present invention as an RNA polymerase inhibitor, *in vivo* and *in vitro* tests may be performed to further characterize the nature and mechanism of the inhibitory

activity. For example, classical enzyme kinetic plots can be used to distinguish, e.g., competitive and non-competitive inhibitors.

Compounds identified as RNA polymerase inhibitors using the methods of the present invention may be modified to enhance potency, efficacy, uptake, stability, and suitability for use in pharmaceutical formulations, etc. These modifications are achieved and tested using methods well-known in the art.

The present invention is further described in the following examples which are intended to further describe the invention without limiting the scope thereof.

Example 1: **Isolation and Analysis of the *M. tuberculosis rpoA* Gene**

The following experiments were performed in order to clone the gene encoding *M. tuberculosis* RNA polymerase α subunit. In these experiments, the following materials and methods were used.

A. Materials and Methods

PCR amplification: *M. tuberculosis* H37Rv genomic DNA (1 ng) was amplified in a 100 µl reaction containing: 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 500 mM KCl, 2.5 U Taq polymerase (Beohringer-Mannheim), 0.2 mM dNTPs and oligonucleotide primers (1 µM each): 5'-CTACGCAAGCAGGGTCCGGGTGAG-3' and 5'-CTCACCCGGACCCTGCTTGCGTAG-3'. PCR was performed in an Omnigene thermocycler with 35 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 1 min. The amplification product was gel-purified and ligated into vector pCR2.1 (Invitrogen).

Cosmid hybridizations: A transducing lysate of a cosmid library of *M. tuberculosis* H37Rv genomic DNA in vector pYA3060 was used. Cosmid-bearing *E. coli* Φ2819T (Jacobs et al., *Infec.Immun.* 52:101, 1986) colonies representing roughly five genomic equivalents were individually picked to wells of sterile 96-well microliter dishes and propagated at 30°C in Luria broth containing ampicillin at 30 µg/ml and thymidine at 50 µg/ml. Colonies were grown overnight at room temperature on the above media as nylon filter replicas of the library. Filters were processed for colony hybridization by standard methods and probe hybridizations performed as described below. Cosmid DNAs were purified using maxiprep columns (Qiagen).

Southern blot analysis: Restriction enzyme digests of *M. tuberculosis* H37Rv chromosomal or cosmid DNAs were resolved by electrophoresis on 1% TAE-agarose gels and blotted to nylon filters using standard methods. Probe labeling and

hybridizations were performed using the Gene Images hybridization system (Amersham) essentially as described by the supplier except that hybridizations and washes were carried out at 70°C.

DNA sequencing and analysis: Plasmid templates for nucleotide sequencing were purified using RPM miniprep kits (Bio101). PCR cycle sequencing (ABI Prizm) was carried out with an Applied Biosystems automated sequencer.

B. Strategy for *M. tuberculosis rpoA* gene isolation

A fragment of the *M. tuberculosis rpoA* gene was isolated by PCR, using oligonucleotide primers homologous to defined regions of α that are conserved among Gram-positive and Gram-negative bacterial species. Amino acid sequence alignments of the α subunits of *B. subtilis* and *E. coli* revealed several short regions of identity clustered primarily within the N-terminal half of α implicated in association with RNA polymerase core subunits. Primers corresponding to conserved α segments LRRILLSS and VTAADITHD (amino acids 39-46 and 106-114, respectively of the *B. subtilis* subunit) specifically amplified a DNA fragment of anticipated size (228 bp) using *M. tuberculosis* H37Rv genomic DNA as a template. To confirm that the amplified DNA contained *rpoA* sequences, the 228 bp fragment was subcloned and a partial nucleotide sequence determined. The deduced amino acid sequence of the PCR product showed significant homology to the α subunits of other bacteria (Figure 1), confirming its identity as an *rpoA* gene fragment. One ORF of the 228 bp fragment displayed high degree of identity to corresponding intervals of the *B. subtilis* (61%) and *E. coli* (55%) α subunits. The *M. tuberculosis rpoA*-derived sequence had an overall G+C content of 60% while the G+C content for bases occupying the third position of each codon increased to 76%, values in good agreement with compositions of other *M. tuberculosis* genes (Weiden et al., in *Tuberculosis*, Little, Brown, 1996, pp. 211-222).

C. Southern blot analysis and isolation of the entire *M. tuberculosis rpoA* gene

To establish that the cloned 228 bp fragment was mycobacterial in origin, Southern blot analysis of restriction enzyme digests of *M. tuberculosis* H37Rv genomic DNA was performed. The PCR probe detected unique restriction fragments in chromosomal digests (Figure 2B). These data are consistent with a single copy of the *rpoA* gene in the *M. tuberculosis* chromosome (two bands of hybridization observed in the

*Eco*RI digest result from the presence of an *Eco*RI site within the 228 bp probe sequence). The full-length *rpoA* gene was obtained from a cosmid library of *M. tuberculosis* H37Rv genomic fragments using the 228 bp PCR probe. Screening of approximately 600 cosmid-bearing *E. coli* colonies (representing roughly 5 genome equivalents) with the *rpoA* gene fragment yielded 4 positive clones. Restriction analysis indicated that the 4 isolates differed from one another with respect to total insert size, though all shared a roughly 10 kb *Not*I fragment which hybridized to the *rpoA* gene probe (Figure 2A). One *rpoA*-hybridizing cosmid clone, designated IC5, was chosen for further analysis. Southern blotting of IC5 DNA digested with a panel of restriction enzymes confirmed that the no gross structural rearrangements of the *rpoA* gene had occurred during cloning (Figure 2B). The 1.4 kb SacII and 1.3 kb SalI *rpoA*-hybridizing fragments of cosmid IC5 were subcloned into vector pSKII+ prior to nucleotide sequencing.

D Sequence analysis of the *M. tuberculosis rpoA* gene

Nucleotide sequencing was performed on plasmid subclones and on cosmid IC5 DNA. Nucleotide and deduced amino acid sequences of the *rpoA* gene of *M. tuberculosis* are shown in Figure 3. The sequence encodes a 1044 bp ORF which has an overall G+C composition of 63% (85% for bases occupying the codon third position). Assuming that the ATG at position 85-87 serves as the initiator codon, the ORF is expected to encode a protein of 347 amino acids. A strong match with the consensus sequence for an *M. tuberculosis* ribosome binding site (GAAAGGA), (Novick, in *Tuberculosis*, Little, Brown, 1996, pp. 187-198) is positioned just upstream of the putative ATG codon. Examination of more than 80 bp of nucleotide sequence upstream of the translation start site did not reveal regions of exact identity with prokaryotic promoter sites. Among α subunits studied in other bacterial species, the deduced amino acid sequence of the 347 residue *M. tuberculosis* protein displayed greatest similarity (48% identity) to the α subunit of the Gram-positive sporulating bacterium *B. subtilis* (Figure 4).

Example 2: Expression of *M. tuberculosis* RNA Polymerase α Subunit and Reconstitution of Active *M. tuberculosis* RNA Polymerase

The following experiments were performed to purify *M. tuberculosis* RNA polymerase α subunit that had been expressed in *E. coli* and to reconstitute enzymatically active RNA polymerase holoenzyme.

A. Methods:

Purification of M. tuberculosis α subunit: Recombinant α was produced in *E. coli* BL21(DE3) cells (Novagen) and grown at 37°C in LB medium containing 25 μg/ml/ kanamycin. Overnight cultures (100 ml) were diluted 10-fold and grown in LB medium containing 25 μg/ml kanamycin until A_{600} = 0.6 (approximately 75 min). Expression of α was induced for 3 hr with 4 mM IPTG. Purification of the α subunit was performd essentially as described by Tang, et. al, *Meth.Enzymol.* 273:130, 1996. Briefly, cell pellets were harvested by centrifugation for 10 min At 4°C (3000 x g). The pellet was resuspended in 20 ml buffer B (6 M guanidine HCl, 10 mM Tris (pH 7.9), 500 mM NaCl, 5 mM imidazole) and lysed by sonication. Inclusion bodies were removed by centrifugation at 42,000 x g for 30 min at 4°C. The sample was then adsorbed onto Ni^{2+}-NTA (Qiagen) in buffer B. The sample was washed twice with 20 ml buffer B; washed twice with buffer B containing 30 mM imidazole; and eluted with 10 ml buffer B containing 500 mM imidazole. Adsorption, washes, and elution were performed with 1 min incubations at 4°C with gentle mixing.

Reconstitution of α with other M. tuberculosis subunits to form core/holo-RNA polymerase: Reconstitution of core/holo- enzyme was performed by combining 60, 300, and 600 μg of α, β, and β', respectively, followed slow removal of the denaturant by dialysis. The combined protein concentration was adjusted to 0.5 mg/ml with buffer B to prevent aggregation (Nobuyuki et al., *Meth.Enzymol* 273:121, 1996) and dialysed against buffer E (50 mM Tris (pH 7.9), 200 mM KCl, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM

EDTA, 5 mM β-mercaptoethanol, 20% (v/v) glycerol) overnight at 4°C. Following dialysis, the sample was activated by incubation for 45 min at 30°C and aggregates cleared by centrifugation at 10,000 x g for 10 min at 4°C. The sample was adsorbed onto Ni^{2+}-NTA in buffer F (50 mM Tris (pH 7.9), 0.5 mM EDTA, 5% (v/v) glycerol), and washed three times in buffer F containing 5 mM imidazole, and eluted in buffer F containing 150 mM imidazole. Adsorption and elution were performed by incubating for 45 min at 4°C and washes for 1 min. Each step was followed by centrifugation at 16,000 x g for 2 min at 4°C. Core/holo- enzyme was dialysed in buffer F (50 mM Tris (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 5% (v/v) glycerol) overnight at 4°C.

Purification of M. tuberculosis holoenzyme: Core polymerase and subassemblies were separated from holoenzyme by elution from a MonoQ column (Pharmacia) using a 0.2 - 0.5 M KCl gradient. Fractions containing holoenzyme were pooled and used the *in vitro* transcription assay.

Promoter construction: Recently, mycobacterium promoters have been cloned using a plasmid shuttle vector pSD7 (Das Gupta et al., *J. Bacteriol.* 175:5186, 1993) and in a later study, the strength of these promoters was analyzed (Bashyam et al., *J. Bacteriol.* 178:4847, 1996). Based on these studies two promoters, T125 and T101, identified as a weak and a strong promoter, respectively, were cloned into the pUC19 vector and used as templates for *M. tuberculosis* transcription asays.

Transcriptional activity: Holoenzyme was reconstituted by the addition of primary sigma factor MysA to the core polymerase and incubation at 30°C for 20 minutes. The transcription reaction was performed as described (Shorenstein et al., *J. Biol.Chem.* 248:6170, 1973) except that 3 µg of template (pUC19, pMC116, or pMC117) was used per reaction containing a high salt transcription buffer (50 mM Tris (pH7.9), 10 $MgCl_2$, 200 mM KCl, 10 mM DTT, 0.1 mM EDTA, 1 mM K_2HPO_4 (pH7.5), 100 µg/ml BSA). 50 µl

reactions were incubated for 30 minutes at 37°C. To precipitate the RNA transcripts and to stop the reaction, 100 µl of 10% TCA were added to the reaction. The TCA-precipitated RNA was adsorbed onto UniFilter GF/C (Packard, Meriden, CT) double-thick glass fiber filtermats using a cell harvester (Packard, Meriden, CT). The wells of the microtiter plate and the filter were washed two times with 5% TCA and bound radioactivity was determined using a TopCount-HTS (Packard, Meriden, CT) scintillation counter.

B. Results:

Recombinant *M. tuberculosis* α subunit was overproduced in soluble form in *E coli* cells transformed with plasmid pJH37, a derivative of pET26b, containing the entire *rpoA* coding region. *M. tuberculosis* α was expressed at high levels as a C-terminal hexahistidine-tagged fusion protein and purified to homogeneity by affinity chromatography. The majority of the 6xHis-tagged α subunit was located in the soluble fraction and was further purified using a Ni^{2+}-NTA column. Elution of the α subunit polypeptide was performed using 0.5 M imidazole, resulting in >90% recovery of the subunits with >95% purity as estimated by SDS-PAGE (Figure 5).

Core enzyme was reconstituted by combining crude preparations of the β and β' subunits with α polypeptide that contained a histidine tag, followed by removal of denaturant by dialysis to facilitate refolding. Holoenzyme was reconstituted by combining the core polymerase with purified primary σ (MysA) from *M. tuberculosis*. The core/ holoenzyme, containing a 6xHis tag on the α subunit, was purified from free subunits and subassemblies by batch elution from Ni^{2+}-NTA followed by elution from ion exchange chromatography (Figure 6).

To demonstrate that reconstituted *M. tuberculosis* RNA polymerase possesses transcriptional activity, *in vitro* transcription reactions were performed using MysA-containing recombinant holoenzyme and templates containing two known MysA-dependent promoters (T101 and T125, which correspond to strong and weak mycobacterial promoters, respectively). The transcriptional activity of the reconstituted holoenzyme was more than two-fold higher when T101 was used as template as compared with T125 (Figure 7), suggesting that the rate of transcription was related to both the strength of the promoter and to the particular σ factor used.

These experiments demonstrated that *M. tuberculosis* α subunit produced according to the invention can be purified and reconstituted into enzymatically active RNA polymerase holoenzyme that exhibits specificity for *M. tuberculosis* promoters.

Example 3: <u>High Throughput Screens For Inhibitors of *M. Tuberculosis* RNA Polymerase and α Subunit</u>

High-throughput screens for anti-tuberculosis agents may be performed using either an *in vitro* or *in vivo* format. In either case, the ability of test compounds to inhibit *M. tuberculosis* RNA polymerase-driven transcription of *M. tuberculosis* promoters is tested. Typically, a strong promoter such as T101 is used for the test reactions. The sequence of the T101 promoter is:

T101:5'

AGCTTGCAGATCTAGCGATCGCAGCCGACGTGATACCTGACCGTTGTTGATA

GTGTCGGCGGCT-3'

a) In vitro screens:

The following procedure is used for cell-free high-throughput screening. A Tomtec Quadra 96-well pipetting station is used to add the reaction components to polypropylene 96-well dishes. 5 µl aliquots of test compounds dissolved in DMSO (or DMSO alone as a control) are added to wells. This is followed by 20 µl of the RNA polymerase mixture, which consists of: 10 mM DTT, 200 mM KCl, 10 mM Mg^{+2}, 1.5 µM bovine serum albumin, and 0.25 µg reconstituted RNA polymerase. After allowing the test compound to interact with the RNA polymerase, 25 µl of the DNA/NTP mixture is added, containing: 1 µg template DNA (see above), 4 µM [α-^{32}P]-UTP, and 400 µM each CTP, ATP, and GTP.

After incubation for 30 min at 25°C, the reaction is stopped by addition of 150 µl 10% trichloroacetic acid (TCA). After incubation at room temperature for 60 min, the TCA-precipitated RNA is adsorbed onto double-thick glass fiber filtermats using a Tomtec cell harvester. The wells of the microtiter plate and the filter are washed twice with 5% TCA and bound radioactivity is determined usinjg a Wallac microbeta 1450 scintillation counter. Inhibitory activity due to the test compound is calculated according to the formula:

$$\% \text{ inhibition} = \frac{(\text{cpm}_{\text{positive control}} - \text{cpm}_{\text{sample}})}{\text{cpm}_{\text{positive control}}} \times 100$$

where $\text{cpm}_{\text{positive control}}$ represents the average of the cpm in wells that received DMSO alone, and $\text{cpm}_{\text{sample}}$ represents the cpm in the well that received test compound. Compounds that cause at least 50% inhibition are scored as positive "hits" in this assay.

As an additional control, rifampicin is used at a concentration of 30 nM, which results in a 50-75% inhibition of transcription in this assay.

b) In vivo screen:

M. tuberculosis RNA polymerase subunits (α, β, β', and a particular σ subunit) are expressed in *E. coli* under the control of regulatable promoters by transforming *E. coli* with appropriate plasmids. If the σ^A subunit is expressed, a DNA sequence comprising the T101 promoter is also introduced into the cells to serve as a template for *M. tuberculosis*-specific transcription.

In one embodiment, the T101 promoter sequence is linked to a DNA sequence encoding the xylE gene product, catechol 2, 3-dioxygenase (CDO). When expressed in the *E. coli* cell, CDO converts catechol to 2-hydroxymuconic semialdehyde, which has a bright yellow color (having an absorbance maximum at 375 nm) that is easily detected in whole cells or in crude extracts. The substrate for this enzyme is a small aromatic molecule that easily enters the bacterial cytoplasm and does not adversely affect cell viability.

In a high-throughput format, aliquots of bacterial cultures are incubated in the absence or presence of test compounds, and CDO activity is monitored by measuring absorbance at 375 nm following addition of catechol.

c) Specificity:

Compounds that score as positive in either the *in vitro* or *in vivo* assays described above are then tested for their effect on human RNA polymerase II. Those compounds which do not significantly inhibit human RNA polymerase II will be further developed as potential anti-tuberculosis agents.

Claims:

1. An isolated, purified DNA encoding *M. tuberculosis* RNA polymerase α subunit.

2. A DNA as defined in claim 1, wherein said DNA has a sequence selected from the group consisting of the sequence shown in Figure 3, sequence-conservative variants thereof, and function-conservative variants thereof.

3. A DNA vector comprising the nucleic acid sequence of claim 2 operably linked to a transcription regulatory element.

4. A cell comprising a DNA vector as defined in claim 3, wherein said cell is selected from the group consisting of bacterial, fungal, plant, insect, and mammalian cells.

5. A cell as defined in claim 4, wherein said cell is a bacterial cell.

6. An isolated purified polypeptide comprising a polypeptide encoded by a DNA as defined in claim 2.

7. A method for high-throughput screening to detect inhibitors of *M. tuberculosis* RNA polymerase, said method comprising:

a) providing a mixture comprising

(i) purified *M. tuberculosis* RNA polymerase,

(ii) a DNA template encoding a promoter sequence that is recognized by *M. tuberculosis* RNA polymerase;

b) incubating said mixture in the presence of test compounds to form test samples, and in the absence of test compounds to form control samples, wherein said incubating is performed under conditions that result in RNA synthesis in the control samples;

c) measuring RNA synthesis directed by said *M. tuberculosis*-recognized promoter in said test and control samples; and

d) comparing the RNA synthesis detected in step (c) between said test and control samples;

wherein an RNA polymerase inhibitor is a test compound that causes a reduction in RNA synthesis measured in said test sample relative to RNA synthesis measured in said control sample.

8. A method as defined in claim 7, wherein said purified *M. tuberculosis* RNA polymerase comprises recombinant subunits which are reconstituted to form an enzymatically active holoenzyme.

9. A method for high-throughput screening to detect inhibitors of *M. tuberculosis* RNA polymerase, said method comprising:

a) providing a non-mycobacterial bacterial strain that

(i) has been transformed with a DNA template encoding a promoter sequence that is recognized by *M. tuberculosis* RNA polymerase, and

(ii) expresses enzymatically active *M. tuberculosis* RNA polymerase, wherein said polymerase comprises α, β, β', and one of several σ subunits;

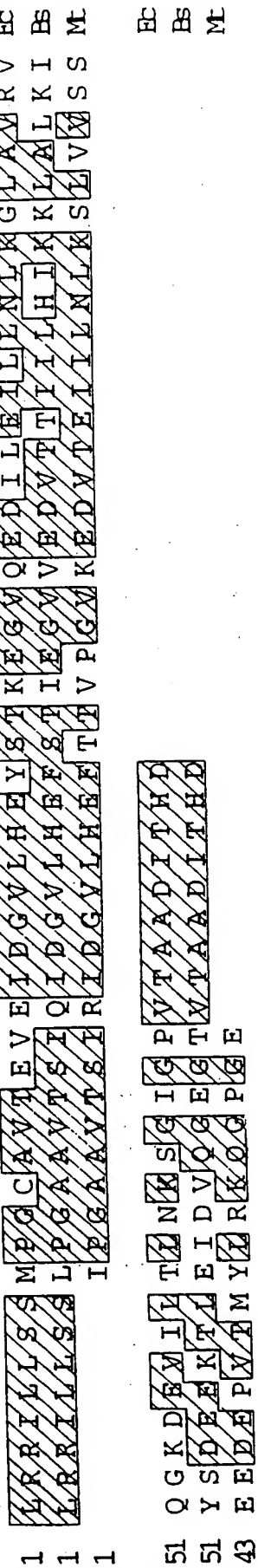
b) incubating the bacterial strain of (a) in the presence of test compounds to form test samples, and in the absence of test compounds to form control samples;

c) measuring RNA synthesis directed by said *M. tuberculosis*-recognized promoter in the test and control samples; and

d) comparing the RNA synthesis detected in step (c) between the test and control samples;

wherein an RNA polymerase inhibitor is a test compound that causes a reduction in RNA synthesis measured in said test sample relative to RNA synthesis measured in said control sample.

FIG. 1



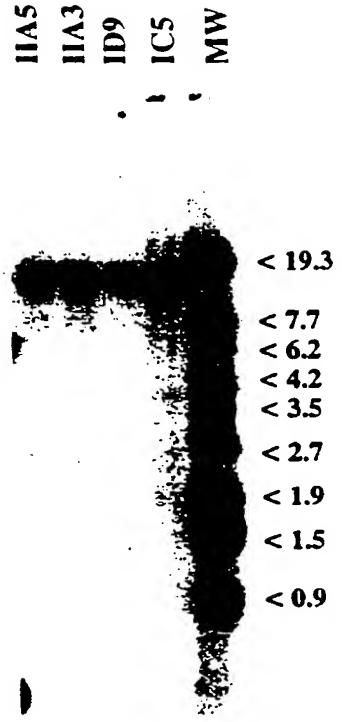


FIG. 2A

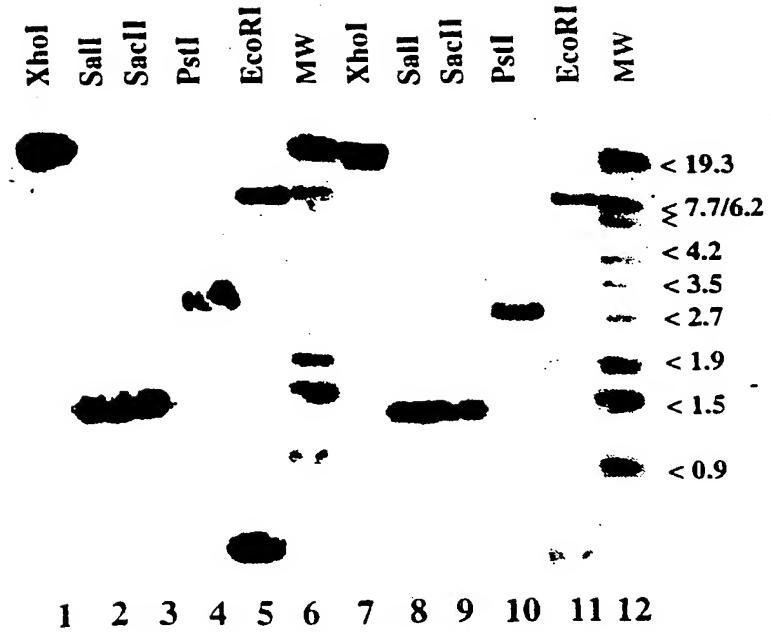


FIG. 2B

FIG. 3A

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   1 cgagtacccccaccttcgggggcgccgcccccgagtgcccccacagacgtcatatggcggacgtcgaaaggaagaagaaa  80

  81 cacc ATG CTG ATC TCA CAG CGC CCC ACC CTG TCC GAG GAC GTC CTC ACC GAC AAC CGA TCC  141
   1      M   L   I   S   Q   R   P   T   L   S   E   D   V   L   T   D   N   R   S   19

 142 CAG TTC GTG ATC GAA CCG CTG GAG CCG GGA TTC GGC TAC ACC CTG GGC AAT TCG CTG CGT  201
  20 Q   F   V   I   E   P   L   E   P   G   F   G   Y   T   L   G   N   S   L   R   39

 202 CGC ACC CTG CTG TCG TCG ATT CCC GGA GCG GCC GTC ACC AGC ATT CGC ATC GAT GGT GTA  261
  40 R   T   L   L   S   S   I   P   G   A   A   V   T   S   I   R   I   D   G   V   59

 262 CTG CAC GAA TTC ACC ACG GTG CCC GGG GTC AAA GAA GAT GTC ACC GAG ATC ATC CTG AAT  321
  60 L   H   E   F   T   T   V   P   G   V   K   E   D   V   T   E   I   I   L   N   79

 322 CTC AAG AGC CTG GTG GTG TCC TCG GAG GAG GAC GAG CCG GTC ACC ATG TAC CTA CGC AAG  381
  80 L   K   S   L   V   V   S   S   E   E   D   E   P   V   T   M   Y   L   R   K   99

 382 CAG GGT CCG GGT GAG GTT ACC GCC GGC GAC ATC GTG CCG CCG GCC GGC GTC ACC GTG CAC  441
 100 Q   G   P   G   E   V   T   A   G   D   I   V   P   P   A   G   V   T   V   H  119

 442 AAC CCC GGC ATG CAC ATC GCC ACG CTG AAC GAT AAG GGC AAG CTG GAA GTC GAG CTC GTC  501
 120 N   P   G   M   H   I   A   T   L   N   D   K   G   K   L   E   V   E   L   V  139

 502 GTC GAG CGT GGC CGC GGC TAT GTC CCG GCG GTG CAA AAC CGG GCT TCG GGT GCC GAA ATT  561
 140 V   E   R   G   R   G   Y   V   P   A   V   Q   N   R   A   S   G   A   E   I  159

 562 GGG CGC ATT CCA GTC GAT TCC ATC TAC TCA CCG GTG CTC AAA GTG ACC TAC AAG GTG GAC  621
 160 G   R   I   P   V   D   S   I   Y   S   P   V   L   K   V   T   Y   K   V   D  179
```

A ——— - ——— - ——— - ——— - ——— - ——— - ——— A

FIG. 3B

A ——— A

```
622 GCC ACC CGG GTC GAG CAG CGC ACC GAC TTC GAC AAG CTG ATC CTG GAC GTG GAG ACC AAG  681
180 A   T   R   V   E   Q   R   T   D   F   D   K   L   I   L   D   V   E   T   K    199

682 AAT TCA ATC AGC CCG CGC GAC GCG CTG GCG TCG GCT GGC AAG ACG CTG GTC GAG TTG TTC  741
200 N   S   I   S   P   R   D   A   L   A   S   A   G   K   T   L   V   E   L   F    219

742 GGC CTG GCA CGG GAA CTC AAC GTC GAG GCC GAA GGC ATC GAG ATC GGG CCG TCG CCG GCC  801
220 G   L   A   R   E   L   N   V   E   A   E   G   I   E   I   G   P   S   P   A    239

802 GAG GCC GAT CAC ATT GCG TCA TTC GCC CTG CCG ATC GAC GAC CTG GAT CTG ACG GTG CGG  861
240 E   A   D   H   I   A   S   F   A   L   P   I   D   D   L   D   L   T   V   R    259

862 TCC TAC AAC TGC CTC AAG CGC GAG GGG GTG CAC ACC GTG GGC GAA CTG GTG GCG CGC ACC  921
260 S   Y   N   C   L   K   R   E   G   V   H   T   V   G   E   L   V   A   R   T    279

922 GAA TCC GAC CTG CTT GAC ATC CGC AAC TTC GGT CAG AAG TCC ATC GAC GAG GTG AAG ATC  981
280 E   S   D   L   L   D   I   R   N   F   G   Q   K   S   I   D   E   V   K   I    299

982 AAG CTG CAC CAG CTG GGC CTG TCA CTC AAG GAC AGC CCG CCG AGC TTC GAC CCC TCG GAG 1041
300 K   L   H   Q   L   G   L   S   L   K   D   S   P   P   S   F   D   P   S   E    319

1042 GTC GCG GGC TAC GAC GTC GCC ACC GGC ACC TGG TCG ACC GAG GGC GCG TAC GAC GAG CAG 1101
 320 V   A   G   Y   D   V   A   T   G   T   W   S   T   E   G   A   Y   D   E   Q    339

1102 GAC TAC GCC GAA ACC GAA CAG CTT TAG actgcctctaatccagacaggagcgtcagctatgcccaagcc 1170
 340 D   Y   A   E   T   E   Q   L   *                                               348
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FIG. 4A

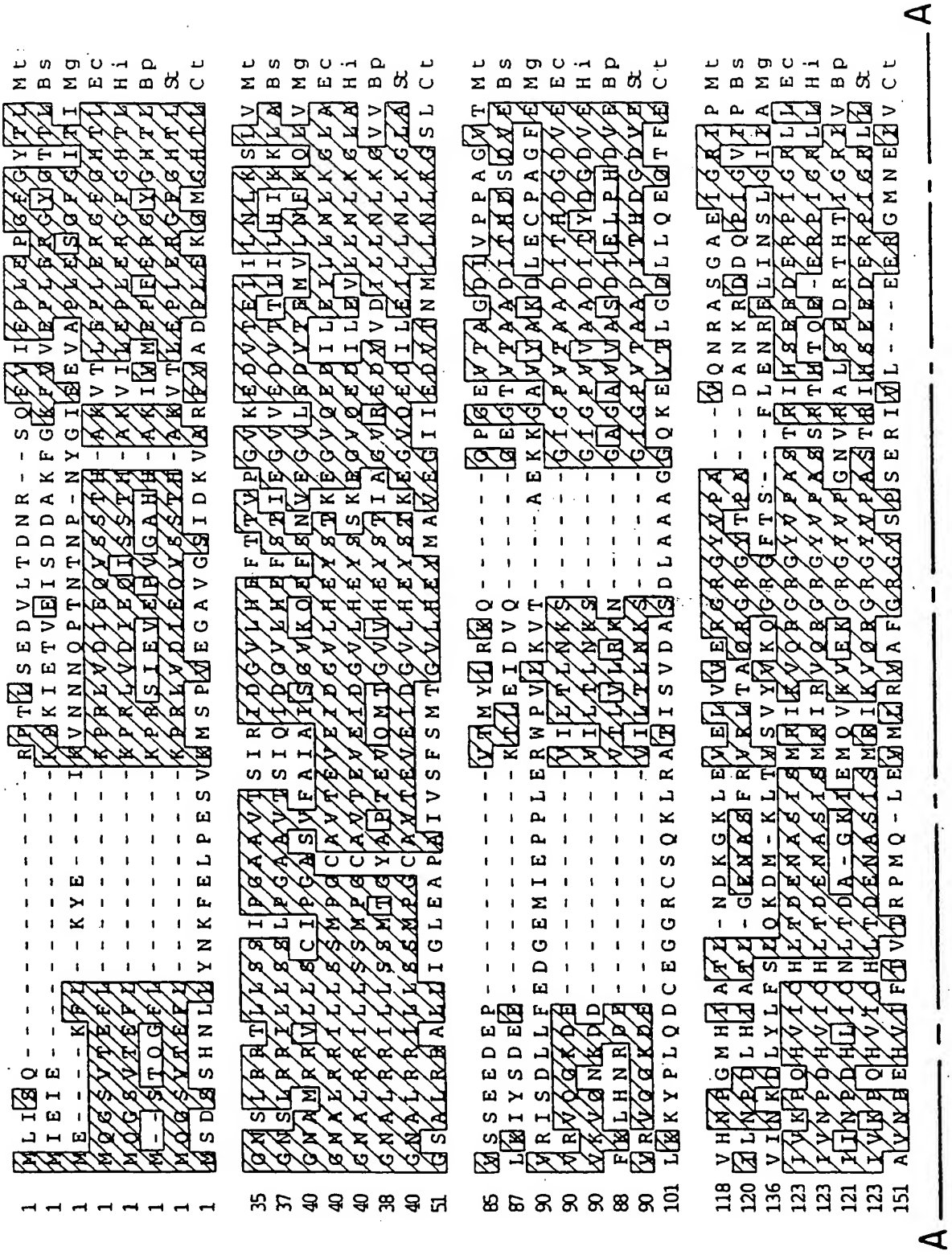


FIG. 4B

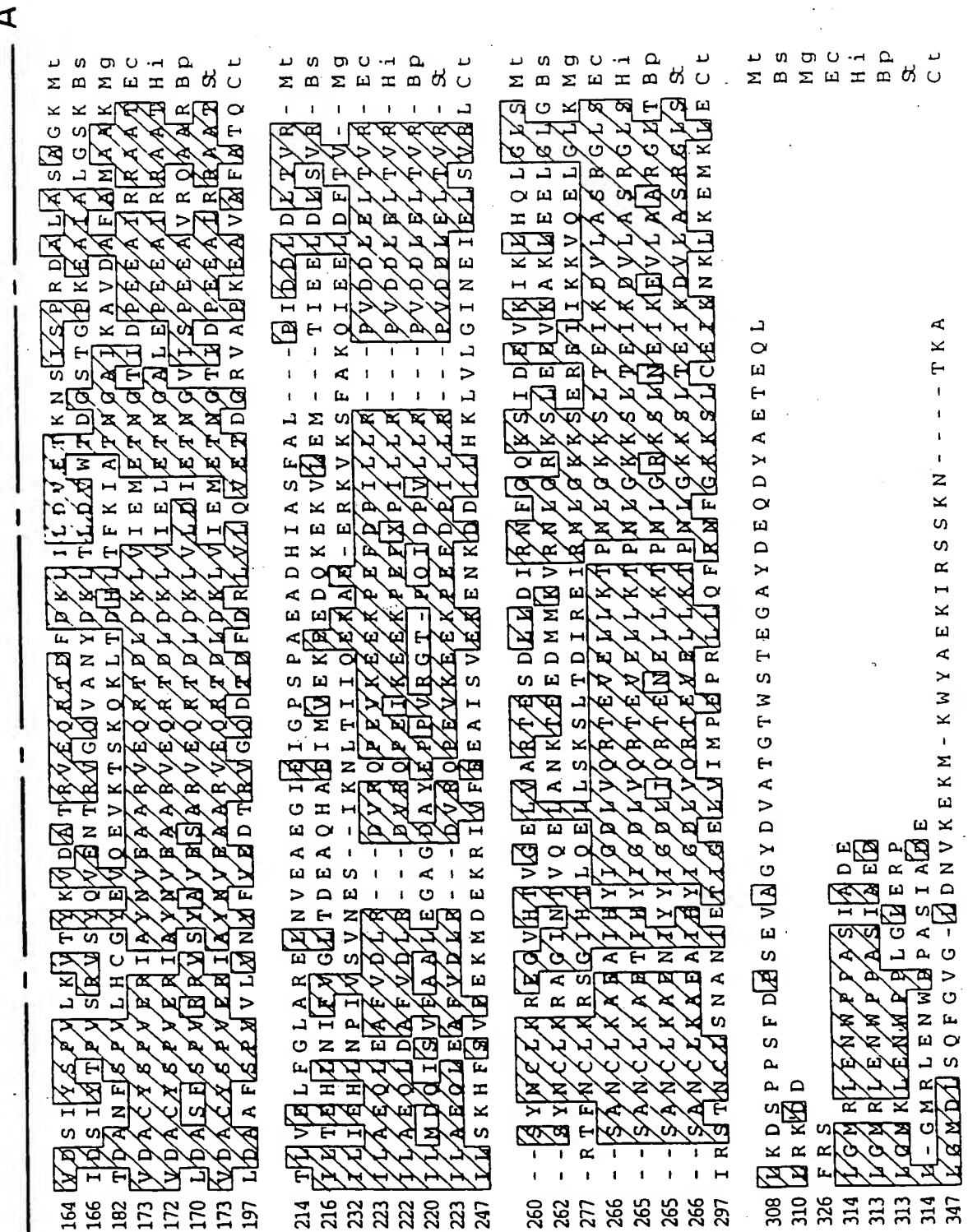


FIG. 5

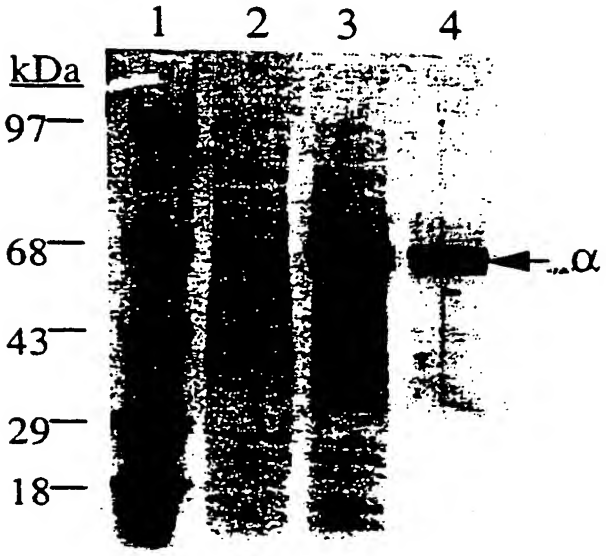


FIG. 6

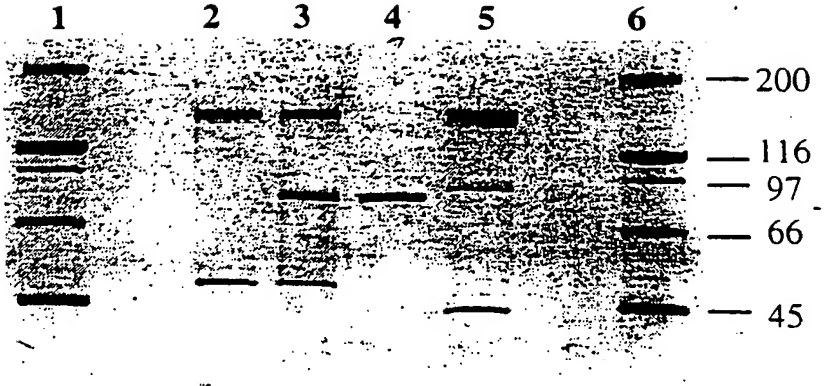
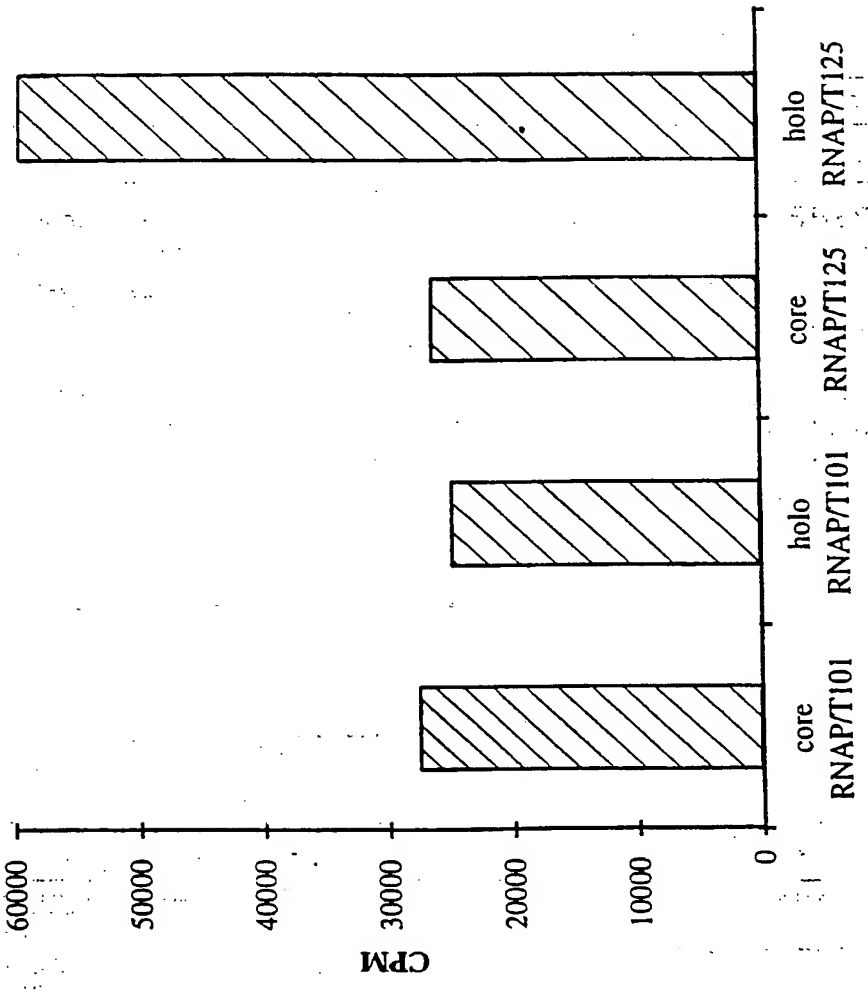


FIG. 7



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/22216

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 9/12, 15/54
US CL :435/194, 15, 325, 419, 252.3, 320.1; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/194, 15, 325, 419, 252.3, 320.1; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG
search terms: RNA polymerase, alpha subunit, Mycobacterium tuberculosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HARSHEY et al. Purification and properties of DNA-dependent RNA polymerase from *Mycobacterium tuberculosis* $H_{37}R_v$. Biochimica et Biophysica Acta. 1976, Vol. 432, No. 1, pages 49-59, see entire document.	1-9
A	MILLER et al. The *rpoB* gene of *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy. April 1994, Vol. 38, No. 4, pages 805-811, see entire document.	1-9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier document published on or after the international filing date
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"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
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"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search 28 JANUARY 1998	Date of mailing of the international search report 23 FEB 1998
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